Claim 69 (new): A method of treating cancer in a subject, said method comprising administering an effective amount of a compound of Claim 53.

Remarks/Arguments

Reconsideration and allowance are respectfully requested in light of this amendment and the following remarks. Applicants have amended the claims to expedite prosecution of the application. This amendment is not intended to acquiesce to the rejections raised by the Examiner and Applicants reserve the right to pursue broader claim subject matter in follow-on applications.

Claims 1-5, 17, 19, 21-23, 25, 27, 29, 31, 33, 36, 38, 40, 43, 45, 47, 49, 51, 53, 57-58, 60 and 62 were rejected under 35 USC §112, second paragraph, as being indefinite, and under 35 USC 112, first paragraph as being overbroad. Claims 36, 38 and 40-42 were rejected under 35 USC 101 and 35 USC 112, first paragraph as being overbroad because claims to broadly treating diseases are not allowed. Claims 14-15, 18, 20, 24, 26, 28, 30, 32, 34, 44, 46, 48, 50, 52, 54, 56, 59, and 61-62 were rejected as being dependent on a rejected claim. Claim 39 was rejected under 35 USC 101 as it was drafted in terms of use. Claims 6-13, 16 and 55 were rejected under 37 CFR 1.141 as containing more than one invention. Restriction between claims 36, 38 and 40 is also requested. Claim 37 was withdrawn from further consideration as it relates to an invention separate from the other claims. Applicants believe that this restriction is in error.

Claims 1-5, 17, 19, 21-23, 25, 27, 29, 31, 33, 36, 38, 40, 43, 45, 47, 49, 51, 53, 57-58, 60 and 62 were rejected under 35 USC §112, second paragraph, as being indefinite, and under 35 USC 112, first paragraph as being overbroad. Specifically, Examiner objects to the terms heterocyclyl and heteroaryl. These terms are defined in detail on pages 81-84 of the specification. Applicants request reconsideration of this rejection as the term is not indefinite or overbroad.

Claims 36, 38 and 40-42 were rejected under 35 USC 101 and 35 USC 112, first paragraph as being overbroad because claims to broadly treating diseases are not allowed. Applicants respectfully disagree. Patent Law requires that a specification disclosure, which contains a teaching of how to make and use the claimed invention in terms that correspond in scope to those used in describing and defining the subject matter, must be taken as in compliance with the enablement requirement under 35 U.S.C. §112, first paragraph, unless there is reason to doubt the objective truth of the statements relied on for enabling support (see MPEP § 2164.02-2164.05).

Applicants respectfully submit that one skilled in the art would understand from Applicants Specification how to make and use the claimed compounds (see Applicants' Specification pages 103-147, 204-296 and 360-394). While working examples are not per se required for enablement, Applicants demonstrated that ~100 compounds of varying structures had at least an IC_{50} of less than 50 nM in *in vitro* models (see Applicants' Specification pages 382-384).

Further, in addition to the compounds made and tested, there is ample support in the art for the credibility of Applicants' invention. Cancer is a group of diseases characterized by dysregulated cell growth control. Growth of cancer cells is dependent on the continued supply of oxygen and nutrients that is delivered to them by vascular networks. Unless neovascularization occurs, tumor growth is limited by the diffusion limit for oxygen and does not progress beyond 1 to 2 mm in size. (P. Carmeliet, Mechanisms of angiogenesis and arteriogenesis, Nature Med., 6:389-395 (2000)). In experimental models of cancer, blocking angiogenesis prevents tumor growth and/or progression. (F.A. Scappaticci, Mechanisms and future directions for angiogenesis-based cancer therapies, J. Clin. Onc., 20:3906-3927 (2002)). The importance of angiogenesis in human cancer is supported by numerous clinicopathologic correlations, which link the production of proangiogenic substances by the cancer cells or the density of microvasculature in tumors to patient prognosis. (See R. Mehta et al., Independent association of angiogenesis index with outcome in prostate cancer, Clin. Cancer Res., 7:81-88 (2001)).

The ability of in vitro endothelial proliferation assays such as that described in the subject application, to support treatment of VEGF-related angiogenesis and cancer claims is understood to one skilled in the art. See, for example: D. Wang et al. Expression and endosytosis of VEGF and its receptors in human colonic vascular endothelial cells. Am. J. Phys. Gast. Live Physiology, 282:G1088-G1096 (2002). W. Auerbach and R. Auerbach, Angiogenesis Inhibition: A review, Pharm. Ther., 63. 265-311, (1994). R. Bagley et al., Endothelial Precursor Cells as a Model of Tumor Endothelium: Characterization and Comparison with Mature Endothelial Cells, Cancer Res., 63:5866-5873 (2003). L. Hennequin et al., Design and Structure-Activity Relationship of a New Class of Potent VEGF Receptor Tyrosine Kinase Inhibitors, J. Med. Chem., 42:5369-5389 (1999). D. Wang et al., Homeostatic Modulation of Cell Surface KDR and Flt1 Expression and Expression of the Vascular Endothelial Cell Growth Factor (VEGF) Receptor mRNAs by VEGF, J. Biol. Chem., 275:15905-15911 (2000). Copies of these references are provided for Examiner's benefit.

Thus Applicants respectfully submit that one skilled in the art would understand from Applicants' Specification how to make and use the compounds of the invention and that Claims 36, 38 and 40-42 meet the requirements of 35 USC 101 and 35 U.S.C. §112, first paragraph. Therefore, Applicants respectfully request that the rejection of the Claims 36, 38 and 40-42 under 35 USC 101 and 35 U.S.C. §112, first paragraph, be withdrawn.

Claim 39 was rejected under 35 USC 101 as it was drafted in terms of use. Claim 39 has been deleted.

Claims 6-13 were rejected under 37 CFR 1.141 as containing more than one invention. Applicants provisionally elect, with traverse, the subject matter of Claim 10.

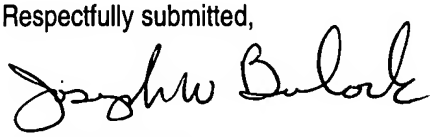
Restriction between claims 36, 38 and 40 is also requested. Applicants provisionally elect, with traverse, the subject matter of Claim 36. However, Applicants believe the Examiner's restriction requirement is improper and should be withdrawn. Applicant's assert there is no undue burden on the Office to search three different indications that the compounds of the claims are useful for treating. Therefore, Applicants respectfully request that the restriction requirement be withdrawn.

Claim 37 was withdrawn from further consideration as it relates to an invention separate from the other claims. Applicants believe that this restriction is in error. One could not manufacture the invention of Claim 37 without making the invention of Claim 1-36, for example. Therefore the inventions are not distinct and withdrawal of Claim 37 is improper. Therefore, Applicants respectfully request that the restriction requirement be withdrawn.

Claims 16 and 55 were rejected under 37 CFR 1.141 as containing more than one invention. Claims 16 and 55 are collections of species of the invention. Applicant's assert there is no undue burden on the Office to search the compounds whether they are in two claims or in 20 claims. Therefore, Applicants respectfully request that the restriction requirement be withdrawn.

It is therefore respectfully submitted that Claims 1-38 and 40-62 are now in condition for allowance. Accordingly, reconsideration and withdrawal of the outstanding rejections, and allowance of Claims 1-38 and 40-62 are respectfully solicited.

Respectfully submitted,



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Expression and endocytosis of VEGF and its receptors in human colonic vascular endothelial cells

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Wang, Dongfang, Richard E. Lehman, David B. Donner, Mary R. Matli, Robert S. Warren, and Mark L. Welton. Expression and endocytosis of VEGF and its receptors in human colonic vascular endothelial cells. *Am J Physiol Gastrointest Liver Physiol* 282: G1088–G1096, 2002.. First published January 2, 2002; 10.1152/ajpgi. 00250.2001.—Normal human colonic microvascular endothelial cells (HUCMEC) have been isolated from surgical specimens by their adherence to Ulex europaeus agglutinin bound to magnetic dynabeads that bind α-L-fucosyl residues on the endothelial cell membrane. Immunocytochemistry demonstrated the presence of a range of endothelial-specific markers on HUCMEC, including the von Willebrand factor, Ulex europaeus agglutinin, and platelet endothelial cell adhesion molecule-1. The growing cells form monolayers with the characteristic cobblestone morphology of endothelial cells and eventually form tube-like structures. HUCMEC produce vascular endothelial growth factor (VEGF) and express the receptors, kinase insert domain-containing receptor (KDR) and fms-like tyrosine kinase, through which VEGF mediates its actions in the endothelium. VEGF induces the tyrosine phosphorylation of KDR and a proliferative response from HUCMEC comparable to that elicited from human umbilical vein endothelial cells (HUVEC). On binding to HUCMEC or HUVEC, ^{125}I-labeled VEGF internalizes or dissociates to the medium. Once internalized, ^{125}I-labeled VEGF is degraded and no evidence of ligand recycling was observed. However, significantly less VEGF is internalized, and more is released to the medium from HUCMEC than HUVEC. Angiogenesis results from the proliferation and migration of microvascular, not large-vessel, endothelial cells. The demonstration that microvascular endothelial cells degrade less and release more VEGF to the medium than large-vessel endothelial cells identifies a mechanism permissive of the role of microvascular cells in angiogenesis.

vascular endothelial growth factor; colon

VASCULAR ENDOTHELIAL CELLS play an important role in a number of physiological and pathological processes including vascular wall homeostasis, blood coagulation, leukocyte adhesion, and angiogenesis, including angiogenesis associated with inflammatory processes, wound healing, and cancer (11, 14–16, 40, 50). Specific, fundamental functions of endothelial cells are mediated by vascular endothelial growth factor (VEGF), which acts directly and relatively selectively on these cells (11, 14–16, 49). VEGF promotes the proliferation, viability, and migration of endothelial cells in vitro and increases vascular permeability and angiogenesis in vivo (6, 11–12, 14–16, 46). In addition, VEGF stimulates glucose uptake and the production of tissue factor, collagenase, and plasminogen activators and inhibitors by endothelial cells. VEGF receptors are largely restricted to endothelial cells. Only a few nonendothelial cell types bind VEGF (1–3, 32, 33).

Two homologous, high-affinity VEGF receptors, kinase insert domain-containing receptor (KDR) and fms-like tyrosine kinase (Flt-1), have been identified on human endothelial cells (8, 34, 35, 38, 39, 47), including human umbilical vein endothelial cells (HUVEC) (56), gastric endothelial cells (30), and human dermal microvascular endothelial cells (HDMEC) (24). KDR and Flt-1 consist of ~1,300 amino acid residues. The receptors are composed of an extracellular region containing seven immunoglobulin-like loops, a short membrane-spanning sequence, and an intracellular, split tyrosine kinase (38, 44, 49). Binding of VEGF to KDR or Flt-1 induces receptor autophosphorylation, although activation of Flt-1 can be difficult to detect, probably due to its relative nonabundance and weak tyrosine kinase activity (38, 55). VEGF binding and receptor activation induce tyrosine phosphorylation of cytoplasmic signaling proteins that contain SH2 domains (22). These proteins allow KDR and Flt-1 to communicate with signaling pathways that promote responses to VEGF.

Numerous cell surface receptors undergo endocytosis after formation of a ligand-receptor complex. An acidic environment in endosomes promotes dissociation of ligand/receptor complexes. Subsequently, the ligand, the receptor, or both, can recycle to the cell surface or undergo degradation in lysosomes (7, 37, 53). Rela-

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tively little is known of the mechanisms through which endothelial cells process VEGF. In one study, Dougher and Terman (9) found that VEGF is internalized by bovine aortic endothelial cells and human embryonic kidney epithelial cells transfected with KDR and that KDR activation is required not only for the induction of cellular responses but for receptor internalization and signal termination as well (9). However, considerable heterogeneity exists, both structurally and functionally, among endothelial cells from different organs and between the endothelium in large vessels and microvessels. Endothelial cells may differ in morphology, endothelial markers, response to growth factors, basal release of endothelial cell-derived factors, and even susceptibility to invasion by metastatic tumor cells (36). Thus it is important to identify the characteristics of the endothelial cells in specific organs that perform distinct processes and are subject to attack by distinct pathologies.

In a previous study with human large-vessel endothelial cells (HUVEC), we found that VEGF induced downregulation of both VEGF receptors (56). However, concomitant with downregulation of the receptor proteins, the mRNAs for both receptors were upregulated. These observations indicated that as VEGF action is important to endothelial cell survival (57), these cells are protected against inappropriate or prolonged loss of VEGF receptors (56). These results illustrate the importance of VEGF receptor expression to the endothelium and suggest that how endothelial cells treat VEGF would be comparably important.

As a first step in a long-term study of angiogenesis in colon cancer and inflammatory diseases, microvascular endothelial cells from human colon (HUCMEC) were isolated. HUCMEC express VEGF, KDR, and Flt-1 and are responsive to VEGF. Comparative studies show that HUCMEC and HUVEC process VEGF differently. Rather than internalizing and degrading most VEGF that binds the cells, HUCMEC release intact, active VEGF to the medium where it can support angiogenesis through autocrine and paracrine mechanisms.

MATERIALS AND METHODS

Reagents. Collagenase type II was from Worthington Biochemical (Freehold, NJ). Ulex europaeus agglutinin-1 (UEA-1) unconjugated and conjugated with fluorescein or rhodamine and Vectashield were from Vector (Burlingame, CA). M-450 tosyl-activated dynabeads and the magnetic particle concentrator were from Dynal (Oslo, Norway). Antibodies to von Willebrand factor (vWF), cytokeratin (CK5), mouse IgG (H+L), and F(ab')$_2$-FITC were from Boehringer-Mannheim (Indianapolis, IN). Anti-platelet endothelial cell adhesion molecule-1 (anti-PECAM-1; CD31) was from Collaborative Biomedical Products (Bedford, MA). Acetylated low-density lipoprotein labeled with 1,1'dioctadecyl-3,3,3',3',-tetramethylindo-carbocyanine perchlorate (DiI-Ac-LDL) was from Biomedical Technologies (Stoughton, MA). Recombinant human VEGF$_{165}$ was a gift from Dr. Napoleone Ferrara (Genentech, South San Francisco, CA).

Isolation and culture of colonic endothelial cells. UEA-1 lectin was bound to magnetic dynabeads (18, 30). Briefly, 250 μl of UEA-1 (0.2 mg/ml in 0.5 M borate buffer, pH 9.5) and 250 μl of dynabeads (4×10^8 beads/ml) were rotated overnight at room temperature. The beads were collected with a magnet, washed three times for 15 min at 4°C with Hank's balanced salt solution (HBSS) containing 5% fetal bovine serum (FBS), and finally washed overnight. The beads were collected with a magnet and suspended at a 4×10^8 beads/ml in HBSS containing 5% FBS.

Macroscopically normal colon was taken from the proximal margin of colectomies performed for malignancy. After mesentery was removed, the remaining tissue was placed in ice-cold PBS containing 10,000 U/ml of penicillin, 10,000 μg/ml streptomycin sulfate and 25 μg/ml of amphotericin B, and cut into ~5-mm cubes. The diced tissue was pelleted, suspended in 0.25% collagenase type II in PBS, and incubated at 37°C for 30–60 min while being shaken. Digested tissue was washed twice with HBSS/5% FBS, suspended in endothelial growth medium (EGM) (Clonetics, CA), and filtered through a 70-μ cell strainer (Falcon, Oxnard, CA). The mixed cell population was seeded into T-75 flasks coated with 2% gelatin and maintained at 37°C in 5% CO_2 for 24 h. Adherent cells were washed three times with HBSS/ 5% FBS and maintained in culture for 2–5 days. To isolate endothelial cells, the cells were trypsinized to allow resuspension and added to 180 μl HBSS/5% FBS, mixed with 20 μl of UEA-1 conjugated beads, and shaken for 15 min at room temperature. The beads were added in excess to give a ratio of ~20 beads per cell. The cell-bead mixture was suspended in 5 ml HBSS/5% FBS and gently agitated. Beads with attached endothelial cells were concentrated by adherence to a magnet during a collection process that proceeded for at least 1 min. The beads, with the attached endothelial cells, were washed with 5 ml HBSS/5% FBS, a process repeated at least three times. The beads were suspended in EGM, seeded into T-75 flasks coated with 2% gelatin, and grown in a 37°C 5% CO_2 humidified incubator. HUCMEC were studied between *passages 2* and *5*. HUVEC isolated by the method of Jaffe et al. (31) were cultured under the same conditions as HUCMEC.

Immunochemistry. Endothelial cells were cultured in PBS containing 5% normal goat serum and 0.1% saponin for 30 min, and then incubated for 16 h at 4°C with a mixture of antibodies to vWF (2 μg/ml), PECAM-1 (200 μg /ml), and CK5. The cells were washed and incubated for 2 h at room temperature with secondary antibodies conjugated to fluorescein isothiocyanate. To demonstrate DiI-Ac-LDL uptake, living cells were incubated for 16 h at 37°C with EGM containing 10 μg/ml DiI-Ac-LDL. Cells were then fixed by incubation with 4% paraformaldehyde in PBS for 20 min at 4°C. To demonstrate UEA-1 binding, cells were fixed by 1% paraformaldehyde in PBS for 10 min at room temperature and incubated with 20 μg/ml fluorescein-labeled UEA-1 in PBS/5% normal goat serum for 16 h at 4°C. To identify endothelial cells in tissues, segments of human colon were fixed by incubation with 4% paraformaldehyde in PBS (pH 7.4) for 16 h at 4°C. Frozen sections of 10 μm were prepared. Slides were incubated with PBS/5% normal goat serum/0.3% Triton X-100 for 30 min and then incubated with anti-vWF in PBS/5% normal goat serum/0.3% Triton X-100 for 16 h at 4°C. Slides were washed and incubated with an anti-mouse IgG conjugated to fluorescein isothiocyanate for 2 h at room temperature. Endothelial cells were also detected by incubating colon sections with UEA-1 conjugated to rhodamine or fluorescein for 16 h at 4°C.

Assays for VEGF. For Northern blot analysis, total RNA was extracted from near-confluent HUCMEC using RNAzol (Tel-Test, Friendswood, TX). RNA was separated by electrophoresis through 1% agarose-formaldehyde and transferred to nylon membranes. Blots were hybridized to the full-length human VEGF cDNA labeled with [^{32}P]dCTP. For assay of

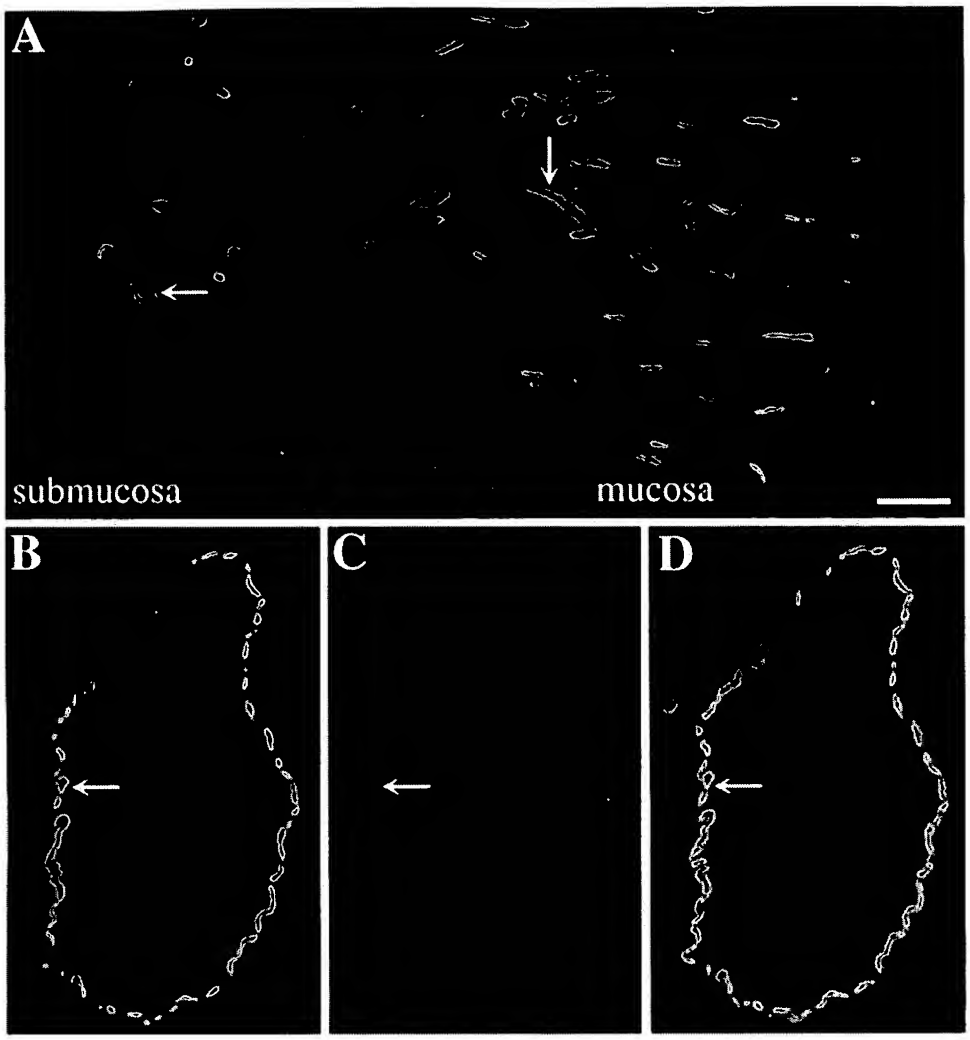

Fig. 1. Characteristics of human colon endothelial cells. *A*: cross section of human colon showing fluorescein-Ulex europaeus agglutinin (UEA)-1 bound to endothelial cells of capillaries, venules, and arterioles throughout the colonic mucosa and submucosa (indicated by arrows); *B*: FITC-labeled vWF localized to the endothelial cells of a venule; *C*: rhodamine-UEA-1 bound to endothelial cells of the same venule; *D*: superimposition of images *B* and *C* showing colocalization (yellow) of UEA-1 and von Willebrand factor (vWF) staining. Scale bar = 100 μm.

VEGF by ELISA, cells were seeded into 10-cm dishes and cultured for 3 days in EGM. Media were collected, centrifuged at 1,200 rpm for 15 min at 4°C, and the supernatant was assayed for VEGF using a Quantikine human VEGF immunoassay kit (R&D Systems, Minneapolis, MN).

Assay of KDR and Flt-1 mRNA by RT-PCR. Total RNA was isolated from HUCMEC or HUVEC, as described above. SuperScript reverse transcriptase and random hexamers (GIBCO-BRL, Gaithersburg, MD) were used to synthesize cDNA. The PCR reaction was carried out in a Perkin-Elmer DNA thermal cycler. For KDR, the sense primer was 5′ACGCTGACATGTACGGTCTAT3′ and the antisense primer was 5′TTCCCATTTGTCGGCATCATA3′. For Flt-1, the sense primer was 5′GCAAGGTGTGACTTTTGTTC3′ and the antisense primer was 5′AGGATTTCTTCCCCTGTGTA3′.

^{125}I-labeled VEGF binding. Recombinant human VEGF was labeled to a specific activity of 167,000 counts·min^{-1}·ng^{-1} using Iodo-Gen (17). For binding assays, duplicate nearly confluent cultures (3.8 cm^2) in binding buffer (HBSS; 25 mM HEPES, pH 7.4, 1 mg/ml BSA) were incubated with ^{125}I-labeled VEGF (1–400 pM) in the absence or presence of a 100-fold excess of VEGF for 6 h at 4°C. Binding of ^{125}I-labeled VEGF by itself is defined as total binding. Binding of ^{125}I-labeled VEGF in the presence of excess VEGF is defined as nonspecific binding. The difference between the total and nonspecific radioactive uptake is specific receptor binding. Cultures were washed with binding buffer and solubilized into 0.1 N NaOH; radioactivity was assayed in a gamma counter. Data were analyzed by the method of Scatchard (42).

KDR phosphorylation. HUCMEC were seeded into 6-cm tissue culture plates. Confluent cells were starved overnight in serum-free 1:1 F12-MEM, stimulated with 50 ng/ml human recombinant $VEGF_{165}$, and lysed into 0.5 ml lysis buffer of (in mM): 50 HEPES, 150 NaCl, 1 EGTA, 50 NaF, 1 PMSF, 2 Na_3VO_4, plus 1% Triton X-100, 10% glycerol, 1 mg/ml aprotinin, and 1 mg/ml leupeptin. Lysates were centrifuged (10,000 *g*, 5 min), and the supernatants were incubated with 4 μg/ml of rabbit anti-KDR (kindly provided by Dr. Harald App, Sugen, CA) for 16 h at 4°C and then with 20 μl of protein G Sepharose (Pharmacia) for 4 h. Sepharose/immune complex conjugates were collected by centrifugation, washed three times with lysis buffer, and boiled for 3 min in 40 μl of 2× sample buffer. After centrifugation, proteins in the supernatants were fractionated by electrophoresis on 7.5% polyacrylamide gels and transferred to Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham). The membranes were blocked with 3% milk powder-TBS [20 mM Tris·HCl (pH 7.6) and 137 mM NaCl] for 1 h at room temperature. Monoclonal phosphotyrosine antibody PY-20 (1:1,500) and goat anti-mouse IgG HRP conjugate (1:2,000) were used to detect phosphotyrosine.

VEGF trafficking. ^{125}I-labeled $VEGF_{165}$ internalization, release, degradation, and recycling were assayed as described (4, 13) with modifications. Briefly, 1 × 10^5 HUVEC or

HUCMEC were seeded into 24-well plates and cultured overnight. After washing with serum-free MEM, the cells were incubated with 400 pM ^{125}I-labeled VEGF in serum-free MEM/0.1% BSA under the conditions indicated in the figure legends. The cells were washed three times with ice-cold PBS (pH 7.4) or MEM. Surface-bound ^{125}I-labeled VEGF was dissociated by incubation of cells in ice-cold pH 2-PBS (2 times, 1 min for each dissociation cycle) and then assayed in a gamma counter. Internalized ^{125}I-labeled VEGF was measured by dissociating cell surface-bound ligand and then assaying the remaining radioactivity in cells solubilized into 0.2 N NaOH. For assessing the integrity of ^{125}I-labeled VEGF released from cells to the medium, cells were washed three times with ice-cold, serum-free MEM and then cultured in serum-free MEM at 37°C. At the indicated times, medium was collected, and trichloroacetic acid and BSA were added to final concentrations of 10 and 0.1%, respectively. Intact ^{125}I-labeled VEGF precipitated in the trichloroacetic acid, whereas degraded ^{125}I-labeled VEGF did not. After centrifugation (15,000 *g*, 10 min), degraded ^{125}I-labeled VEGF was in the supernatant, and intact ^{125}I-labeled VEGF was in the precipitate.

RESULTS

Isolation and characterization of colonic endothelial cells. We tested whether HUCMEC bind UEA-1 by analyzing sections of human colon incubated with fluorescein-labeled UEA-1. Fluorescein-labeled UEA-1 bound to endothelial cells of capillaries, venules, and arterioles throughout the submucosa, muscularis, and subserosa (Fig. 1*A*). To confirm that cells labeled with UEA-1 were endothelial cells, sections were incubated with vWF antibody. Fig. 1, *B-D*, illustrates that cells that interacted with UEA-1 also interacted with anti-vWF. Thus UEA-1 binds specifically to HUCMEC, suggesting the utility of UEA-1-coated magnetic beads for the isolation of such cells.

To isolate HUCMEC, cells from normal human colon were incubated with UEA-1-coated magnetic beads. The beads bound endothelial cells (Fig. 2*A*), allowing isolation of 1×10^6 endothelial cells/g of tissue. When these cells were cultured and grown to confluence, they displayed a "cobblestone" morphology characteristic of endothelial cells (Fig. 2*B*). The isolated cells were assayed for endothelial-specific properties. Fluorescein-labeled UEA-1 bound the plasma membrane of isolated HUCMEC (Fig. 2*C*). Strong surface fluorescence continued to be detected on HUCMEC through at least eight passages. Antibodies to vWF stained perinuclear vesicles in HUCMEC (Fig. 2*D*), and anti-PECAM-1, an integral membrane glycoprotein located at the intercellular junctions of endothelial cells (26), reacted with the cell surface (Fig. 2*E*). Ac-LDL labeled with 1,1′dioctadecyl-3,3,3′,3′,-tetramethylindo-carbocyanine-perchlorate was taken up by HUCMEC and concentrated in discrete intracellular granules (Fig. 2*F*). After extended culture, HUCMEC spontaneously formed tube-like structures, a process that requires cell migration and proliferation (data not shown). Our observations show that HUCMEC retain various

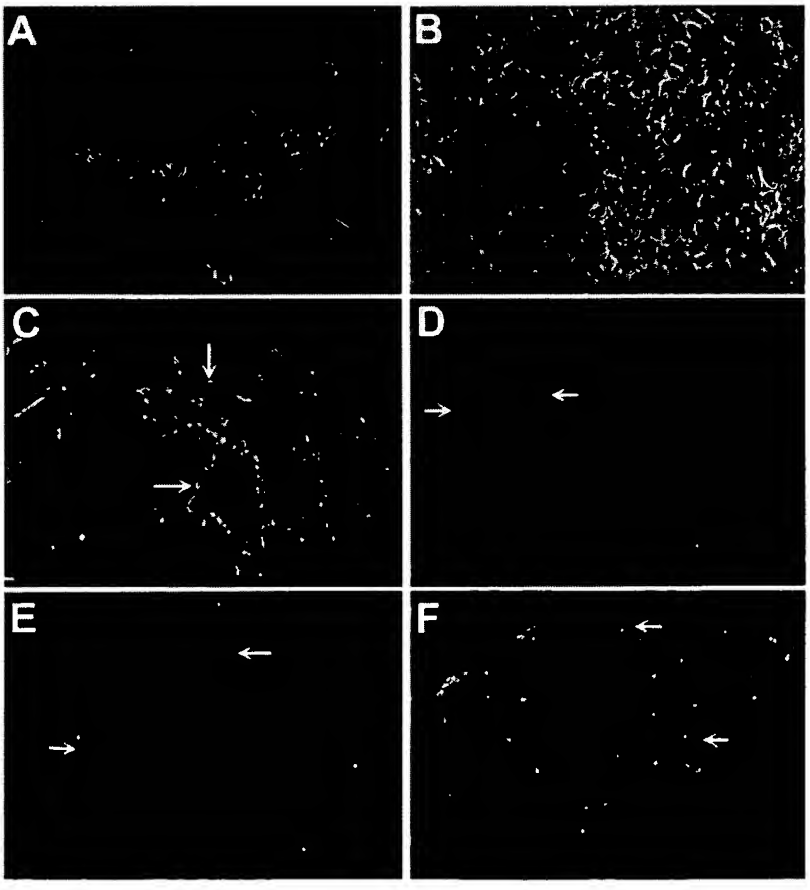

Fig. 2. Human colonic microvascular endothelial cells (HUCMEC) display properties characteristic of endothelial cells. *A*: HUCMEC bound UEA-1-coated magnetic beads; *B*: confluent HUCMEC assume a cobbestone morphology; *C*: HUCMEC stained at the plasma membrane with fluorescein conjugated UEA-1; *D*: immunoreactive vWF was in perinuclear vesicles of HUCMEC; *E*: immunoreactive platelet endothelial cell adhesion molecule-1 was on the surface of HUCMEC; *F*: DiI-Ac-LDL was in discrete granules after uptake by HUCMEC. Arrows indicate specific properties.

endothelial cell properties after isolation from human colon.

HUVEC tested positively for each of the endothelial cell-specific properties displayed by HUCMEC and KNRK cells (rat kidney epithelial cells) negatively (data not shown). On the other hand, HUCMEC and HUVEC did not recognize an antibody to CK5, whereas KNRK cells were stained (data not shown). These results exclude the possibility that HUCMEC were contaminated with mesothelial or epithelial cells.

VEGF expression. We examined HUCMEC for expression of VEGF mRNA by Northern blot analysis and VEGF activity by ELISA. A transcript of 4.4 kb, which corresponds to the size of VEGF mRNA in colon carcinoma cell lines, was detected in HUCMEC from three patients (Fig. 3). VEGF mRNA was also detected in HUVEC. An ELISA of medium conditioned by HUCMEC for 72 h contained 401.4 ± 120.8 pg/ml (range 100–900 pg/ml) of VEGF. Thus cultured HUCMEC express and secrete VEGF.

VEGF receptor expression. VEGF receptors were detected by receptor binding assays with ^{125}I-labeled VEGF. Scatchard analysis of saturation binding assays yielded curvilinear plots. The binding resolved into high affinity (K_D 130 ± 14 pM; 79,900 ± 6,350 sites/cell) and lower affinity (K_D 675 ± 66 pM; 231,300 ± 27,900 sites/cell) sites (Fig. 4*A*). These values are comparable to those obtained in studies with HUVEC (55). RT-PCR was performed to determine whether the binding assays resulted from expression of KDR and Flt-1 by HUCMEC. Amplification with KDR and Flt-1-specific primers generated a 1,100 bp and a 510 bp product, respectively, from HUCMEC cDNA (Fig. 4*B*). The identity of the products from RT-PCR was confirmed by sequence analysis.

Receptor function. An MTT assay showed that VEGF augmented HUCMEC proliferation about twofold after 2 days of incubation (data not shown), a value comparable to that elicited from HUVEC by VEGF, which we reported previously (57). Incubation of HUCMEC with 50 ng/ml VEGF transiently augmented the tyrosine phosphorylation of KDR fourfold after a 10-min incubation at 37°C (Fig. 4*C*). As in our previous studies with HUVEC, Flt-1 tyrosine phosphorylation was undetectable. However, the antibodies to Flt-1 presently available are not recommended for immunoprecipitation, and Flt-1 is less well expressed by endothelial cells than KDR/Flk1.

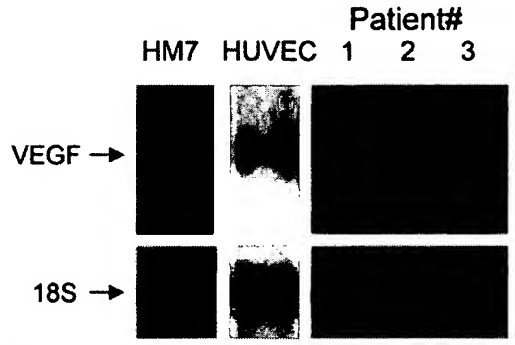

Fig. 3. Northern blot analysis for vascular endothelial growth factor (VEGF). mRNA was isolated from HUCMEC of three patients. A 4.4-kb transcript that corresponded to the size of VEGF in HM7 colon carcinoma cells was detected. 18S ribosome RNA was probed to monitor the amount of total RNA loaded.

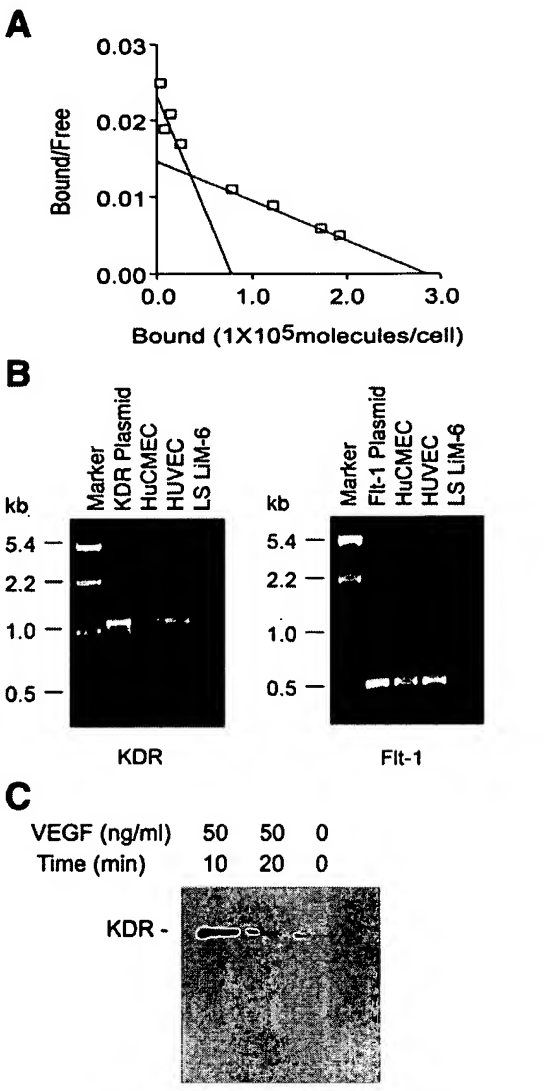

Fig. 4. VEGF receptors of HUCMEC. *A*: Scatchard analysis of the specific binding of ^{125}I-labeled VEGF to near-confluent HUCMEC. *B*: RT-PCR for kinase insert domain-containing receptor (KDR) and fms-like tyrosine kinase (Flt-1) mRNAs. First-strand cDNA was synthesized from total RNA of human umbilical vein endothelial cells (HUVEC) or HUCMEC by reverse transcription. RNA from colon carcinoma cell line LSLiM-6 was a negative control. *C*: VEGF stimulated the tyrosine phosphorylation of KDR. HUCMEC were incubated with 50 ng/ml VEGF. KDR was immunoprecipitated from cell lysates with rabbit anti-KDR. After SDS-PAGE, a Western blot was probed with an anti-phosphotyrosine that detected ~200 kDa KDR.

VEGF processing. The time courses of ^{125}I-labeled VEGF internalization, degradation, recycling, and release by HUCMEC, and HUVEC, were analyzed. In one method, VEGF endocytosis was determined by incubating ^{125}I-labeled VEGF with cells at 4°C for 2 h (Fig. 5, *A* and *C*). At low temperature, internalization and degradation do not take place; consequently, ^{125}I-

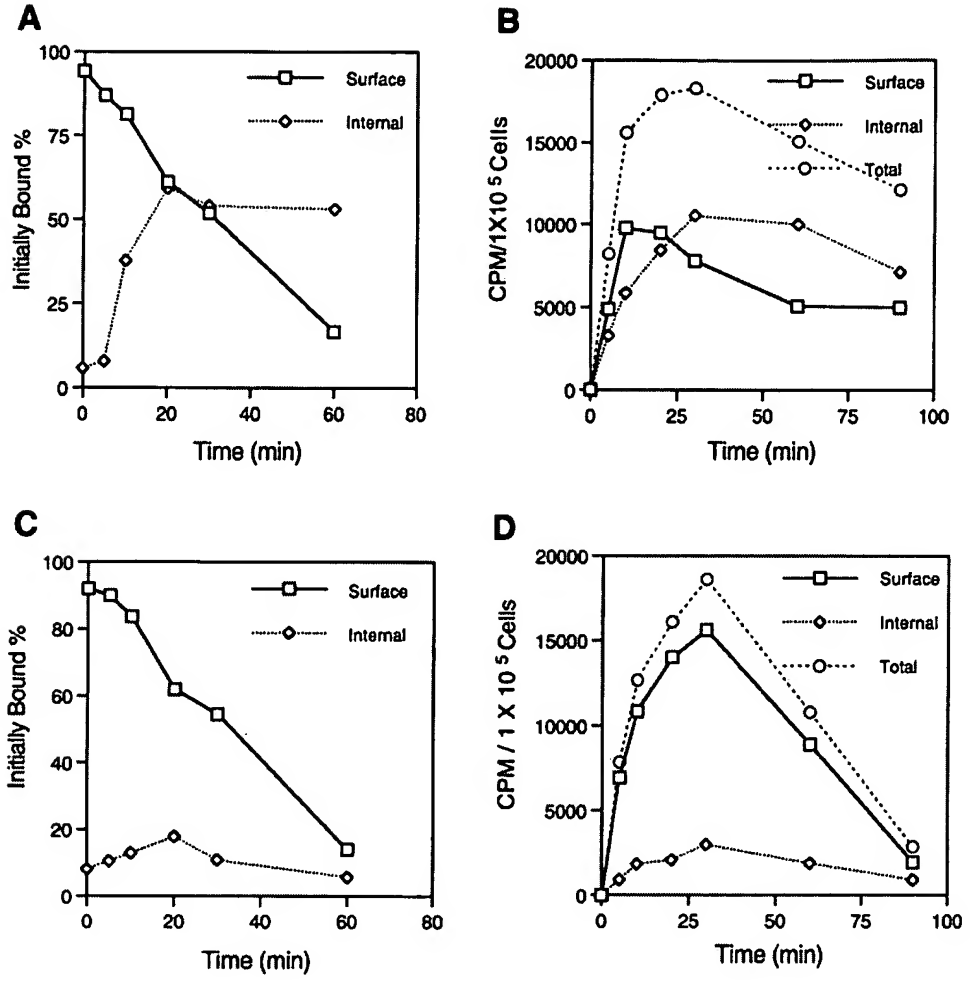

Fig. 5. ^{125}I-labeled VEGF internalization. HUVEC (*A* and *B*) and HUCMEC (*C* and *D*) in 24-well plates were incubated with 400 pM ^{125}I-labeled VEGF at 4°C for 2 h (*A* and *C*) or without the 4°C incubation (*B* and *D*). Cells were transferred to 37°C (*B* and *D*) at indicated time points and washed three times with cold PBS. ^{125}I-labeled VEGF bound to the cell surface was dissociated by washing with pH 2-PBS and assayed in a gamma counter. Internalized VEGF was assayed by measuring radioactivity remaining in the lysates of cells from which surface-bound ^{125}I-labeled VEGF had been dissociated. In *A* and *C*, values are expressed as a percentage of the total cell-associated count at zero time (cpm, counts/min).

labeled VEGF accumulates at the cell surface. A shift of temperature to 37°C initiated internalization indicated by the inability of an acid wash to release ^{125}I-labeled VEGF to the medium. Also, HUVEC or HUCMEC were incubated with ^{125}I-labeled VEGF at 37°C (Fig. 5, *B* and *D*), allowing internalization to initiate immediately. Incubation of HUVEC with ^{125}I-labeled VEGF at 4°C, followed by a temperature shift to 37°C, resulted in maximal internalization after 20 min. At this time, 59% of the ^{125}I-labeled VEGF was internalized. Incubation of HUVEC with ^{125}I-labeled VEGF at 37°C led to maximal internalization after 30 min, at which time 57% of the ^{125}I-labeled VEGF was internalized (Fig. 5, *top*). In experiments with HUCMEC (Fig. 5, *bottom*), maximal internalization after a temperature shift to 37°C occurred after 20 min; incubation initiated at 37°C resulted in peak internalization after 30 min. Thus the time courses over which HUVEC and HUCMEC internalize ^{125}I-labeled VEGF are comparable. However, HUCMEC internalized only about 15% of the bound ^{125}I-labeled VEGF, a much smaller fraction than HUVEC (nearly 60%).

Release of ^{125}I-labeled VEGF to the medium, and its integrity, assayed by SDS-PAGE, was determined. Cells were incubated with ^{125}I-labeled VEGF for 1 h at 20°C to allow binding and internalization before the temperature was shifted to 37°C (Fig. 6, *A* and *B*). The rationale for incubation at 20°C during the first phase of incubation was based on the observation that after incubation at 20°C, internalized ^{125}I-EGF accumulates but is not degraded (13). After 90 min of incubation at 37°C, ~22% of ^{125}I-labeled VEGF bound to HUVEC was released, 47% was internalized, and 20% was degraded. ^{125}I-labeled VEGF on the surface of HUCMEC declined rapidly during incubation at 37°C. This resulted from dissociation of intact ^{125}I-labeled VEGF to the medium rather than internalization and degradation. After 90 min, ~80% of the ^{125}I-labeled VEGF was released, 7% was internalized, and 3% was degraded by HUCMEC. Thus HUVEC degrade more and release less ^{125}I-labeled VEGF than HUCMEC.

In some cell types, the half time for recycling of most membrane components is 10–30 min (37). Thus 60 min of incubation with ^{125}I-labeled VEGF would be sufficient to determine whether internalized ^{125}I-labeled VEGF recycles. ^{125}I-labeled VEGF was bound to HUVEC or HUCMEC at 4°C before the temperature was shifted to 37°C. Surface-bound or internalized ^{125}I-labeled VEGF was then assayed (Fig. 6, *C* and *D*). HUVEC internalized a greater proportion of the ^{125}I-labeled VEGF than HUCMEC. There was no evidence

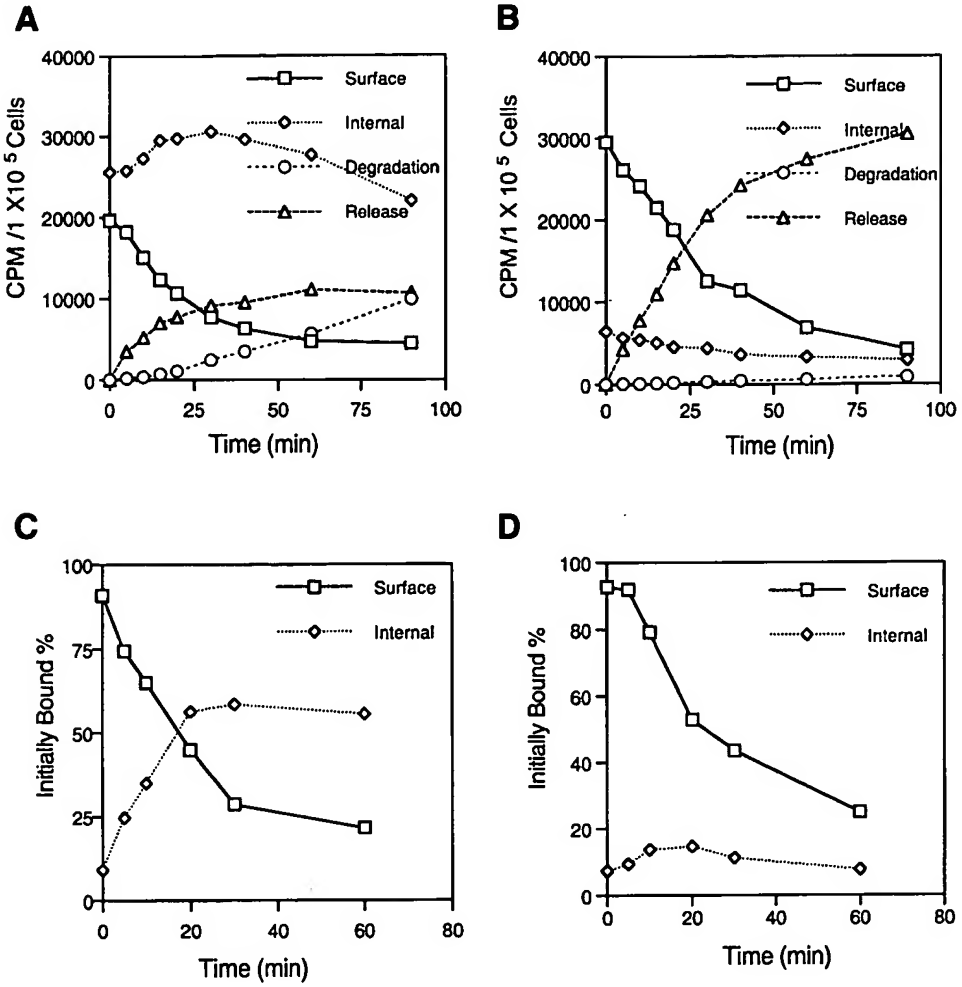

Fig. 6. ^{125}I-labeled VEGF processing. In 24-well plates, HUVEC (*A*) or HUCMEC (*B*) were incubated with 400 pM ^{125}I-labeled VEGF at 20°C for 1 h and then washed 3 times with ice-cold, serum-free MEM. After 0.2 ml of fresh serum-free MEM (37°C) were added, TCA precipitated radioactivity in the medium (intact), TCA soluble radioactivity in the medium (degraded), counts stripped from cell surface at pH 2, and counts remaining in cells were assayed. HUVEC (*C*) or HUCMEC (*D*) were incubated with 400 pM ^{125}I-labeled VEGF at 4°C for 1 h. After plates were washed 3 times with ice-cold, serum-free MEM, 0.2 ml of MEM (37°C) were added, and surface and internalized VEGF were assayed.

for recycling of internalized ^{125}I-labeled VEGF back to the surface of HUVEC or HUCMEC.

DISCUSSION

Endothelium is heterogeneous, and cells isolated from this tissue can be distinguished based on the vessel size and organ within which the vessel resides. Biological characteristics of large-vessel vascular endothelial cells, such as HUVEC, may differ significantly from microvascular endothelial cells. Microvascular endothelial cells sprout from preexisting blood vessels during angiogenesis and are of particular importance to this process (41). We sought to isolate human microvascular colonic endothelial cells (HUCMEC), first for comparison with large-vessel endothelial cells and eventually for the study of angiogenesis in cancer and inflammatory diseases of the colon. It was not possible to isolate HUCMEC by Percoll gradient fractionation or weeding techniques. Magnetic beads coated with the lectin UEA-1, which specifically binds α-L-fucosyl residues on the endothelial cell membrane, were successfully used for isolation. This method has been employed for isolation of endothelial cells from stomach, lung, decidua, and the mammary gland (18, 25, 27, 28, 30). Using this procedure, HUCMEC were isolated from specimens of human colon that maintained morphological characteristics of endothelial cells and endothelial-specific properties.

Expression of VEGF receptors and responsiveness to VEGF are important to endothelial function (11, 15, 16, 38). To understand how VEGF is processed in HUCMEC, we characterized the VEGF receptors of these cells. Scatchard analysis yielded a curvilinear plot that indicated that HUCMEC express two high-affinity VEGF binding sites. These were demonstrated to be KDR and Flt-1 by RT-PCR. Treatment of HUCMEC with VEGF promoted tyrosine phosphoryation of KDR (Fig. 4*C*), which promotes the growth response of HUVEC to VEGF (57). Functionality of KDR on HUCMEC was further indicated by a comparable two-fold increase of HUVEC (57) and HUCMEC proliferation induced by VEGF.

Flt-1 tyrosine phosphorylation could not be detected in control or VEGF-treated HUVEC or HUCMEC. This may be due to the different properties of the KDR and Flt-1 tyrosine kinases, different susceptibilities of these receptors to dephosphorylation by phosphotyrosine phosphatases, or the low level of Flt-1 expression by endothelial cells (23, 38, 55).

EGF, FGF, insulin, IGF-II/mannose-6-phosphate, and glucagon receptors (7, 13, 53, 54) mediate ligand internalization. Internalized ligand may be degraded or recycled to the cell surface, from which it may dissociate or translocate to the nucleus (37, 54). Endocytosis regulates receptor number and cellular responsiveness to ligand, the protein composition of the plasma membrane, remodeling of the cell surface, and the delivery of nutrients into cells (20, 52). We found that internalization and degradation were the predominant mechanisms used by HUVEC to process VEGF. HUCMEC internalize and degrade comparatively little VEGF and release a significantly greater fraction of the mitogen to the medium. The mechanism that underlies the different mode of VEGF processing by HUVEC and HUCMEC is undefined. One possibility is that more VEGF binds to low-affinity heparin sulfate proteoglycans (21) on HUCMEC than on HUVEC. Such interaction might preserve VEGF for release and subsequent interaction with the cells. In support of this possibility are observations that the extracellular domain of KDR contains a binding site for heparin and that Flt-1 shows affinity for heparin (5, 10). However, binding of VEGF-A to Flt-1 and KDR is oppositely affected by heparin; interaction with the former receptor is diminished but binding to the latter is augmented (48). Also, whereas VEGF-A binding to Flt-1 is diminished by heparin, the activity of the receptor is increased (29). Thus the effect of heparin on VEGF binding and function is complex and difficult to assess.

Although endothelial cells from different organs present many common functional and morphological features, they also display remarkable heterogeneity. Even in the same organ, the endothelium of large and small veins and arteries exhibits significant heterogeneity (19). For example, α-thrombin stimulates urokinase production and DNA synthesis in human cerebral microvascular endothelial cells but not in HUVEC (43). Human cerebral endothelial cells respond to vasoactive intestinal polypeptide and endothelin-1 with an increase in intracellular free calcium, whereas omental endothelial cells do not (50, 51). In our study, HUVEC and HUCMEC demonstrated marked differences in VEGF endocytosis. HUCMEC internalize and degrade less and release more VEGF from the cell surface to the medium than do HUVEC, and importantly, released VEGF can be reutilized. These results are consistent with observations showing that VEGF produced by microvascular cells in the neonatal dermis and eyes plays a role in activation of the endothelium (45, 58). It is interesting that microvascular cells, the endothelial cell type involved in angiogenesis, preserve VEGF such that it can again stimulate the cells and, thereby, neovascularization through autocrine and paracrine mechanisms.

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REVIEW ARTICLE

Mechanisms and Future Directions for Angiogenesis-Based Cancer Therapies

By Frank A. Scappaticci

Abstract: **Targeting angiogenesis represents a new strategy for the development of anticancer therapies. New targets derived from proliferating endothelial cells may be useful in developing anticancer drugs that prolong or stabilize the progression of tumors with minimal systemic toxicities. These drugs may also be used as novel imaging and radiommunotherapeutic agents in cancer therapy. In this review, the mechanisms and control of angiogenesis are discussed. Genetic and proteomic approaches to defining new potential targets on tumor vasculature are then summarized, followed by discussion of possible antiangiogenic treatments that may be derived from these targets and current clinical trials. Such strategies involve the use of endogenous antiangiogenic agents, chemotherapy, gene therapy, antiangiogenic radioligands, immunotherapy, and endothelial cell-based therapies. The potential biologic end points, toxicities, and resistance mechanisms to antiangiogenic agents must be considered as these therapies enter clinical trials.**

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ANGIOGENESIS, OR new blood vessel growth from an existing vasculature, has become a very promising target for experimental therapies in cancer, and a wide variety of therapies directed at interfering with this process are in development.[1] The enthusiasm felt by many investigators in the field comes from the potential advantages of such agents compared with standard chemotherapy in treating cancer. These include the easy access to targets within the vasculature, independence of tumor cell resistance mechanisms, and the broad applicability of this therapy to many tumor types.[2] Because angiogenesis is infrequent in the adult, there is the potential to develop very specific therapies with minimal toxicities except during times of wound healing, inflammation, ovulation, pregnancy, or ischemia.[1] An understanding of these various processes and their regulation might lead to differential targeting. Here are described therapeutic strategies that may emerge from angiogenesis research and their potential obstacles when they reach clinical use in man (Fig 1).

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Tumor cells have been understood to be genetically unstable with an ability to mutate and develop resistance to chemotherapeutic drugs.[3] The promise of angiogenesis research has been based on the presumption that endothelial cells supply tumors with a vasculature and, as such, are a stable population of cells that do not change despite mutational events in tumor cells.[4] But are they really? Recent reports suggest that tumor vasculature is quite abnormal and that the endothelial cells that line tumor blood vessels differ in many respects from normal vasculature.[5,6] Blood vessels within a given tumor are heterogeneous and are subject to influences by the local microenvironment and cytokines (such as basic fibroblast growth factor [bFGF], interleukin-8, and interferon alfa) that are produced by tumor cells, macrophages, and other cell types.[7,8] This microenviroment may vary quite extensively when comparing central areas of a bulky tumor, which may be hypoxic and necrotic, with more viable areas toward the periphery.[9] In this regard, endothelial cells within a tumor may be heterogeneous with regions of endothelium that are active, immature, and proliferating in contrast to regions where endothelial cells are quiescent and even undergoing apoptotic processes.[10,11] These observations must be considered when designing vascular-specific therapies.

MECHANISMS OF VASCULOGENESIS AND ANGIOGENESIS

Angiogenesis occurs in a variety of physiologic and pathophysiologic states and is a remodeling of an established primitive network of blood vessels. This is in contrast to vasculogenesis, which is the formation of a primitive network of blood vessels which occurs in embryonic development.[12] An understanding of these two processes and the

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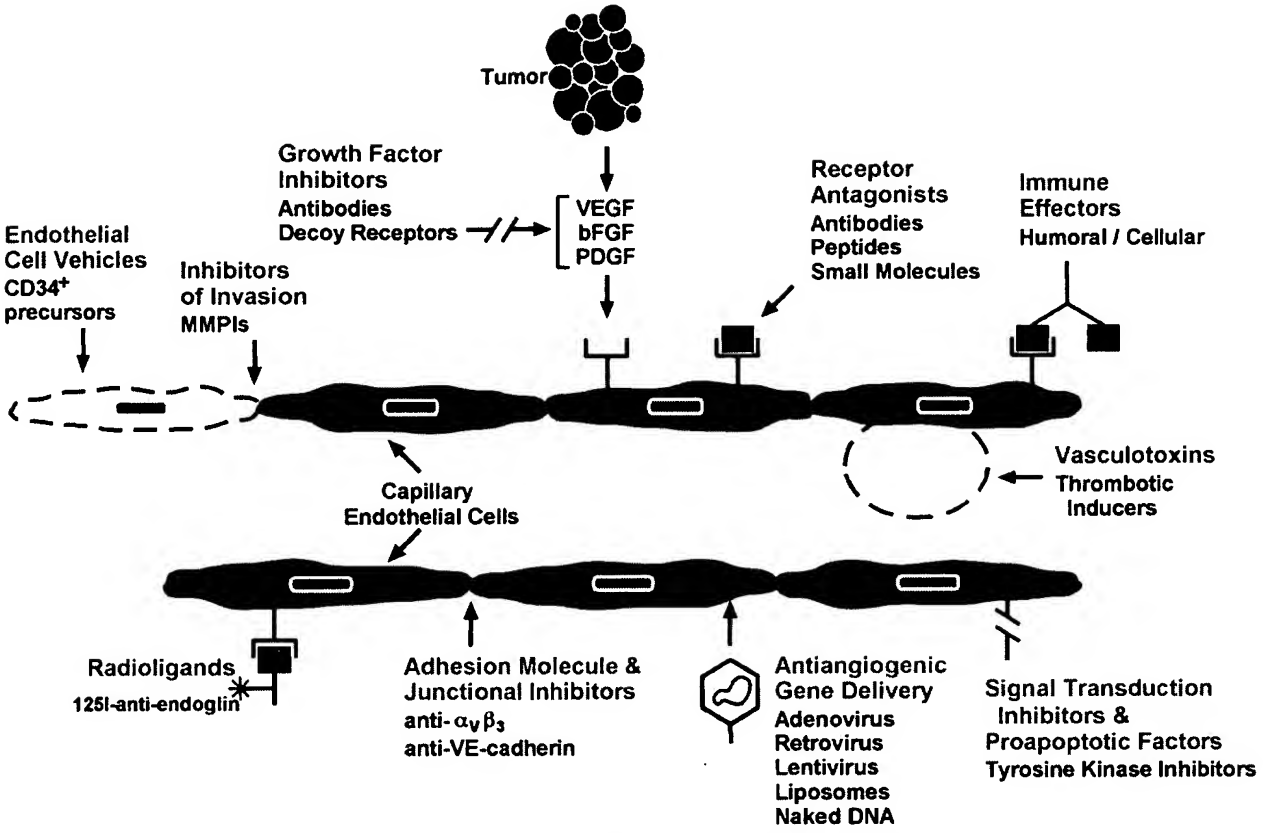

Fig 1. Potential strategies for angiogenesis-based cancer therapies. Scheme for the many different strategies taken to inhibit tumoral angiogenesis using agents that act directly or indirectly on endothelial cells.

factors that regulate growth and differentiation of endothelial cells may provide new clues to methods of intervention for a variety of diseases.

Blood vessels formed during vasculogenesis are derived from mesoderm by differentiation of angioblasts.[13] These angioblasts are endothelial cells that have not formed a lumen and contribute to the formation of primitive vascular networks. Although these processes differ on the nature of the blood vessels and structures that are formed, there are similarities in some of the common growth signals such as vascular endothelial growth factor (VEGF) and fibroblast growth factors.[14-16] These same growth factors also play a role in the development of hematopoietic cells, and this led to suggestions of a precursor hemangioblast. Evidence for this was provided by the VEGF-R2 (also known as Flk-1 in mice and KDR in humans) knockout mouse, which displayed defects in both hematopoietic and angioblastic lineages.[17] The nature of the signals that commit this precursor cell to differentiate into blood cells versus blood vessels is under investigation. On the other hand, the VEGF-R1 receptor was implicated in playing a role in later stages of vascular development because mice lacking this receptor were capable of producing angioblasts, but these cells were unable to assemble into mature vessels.[18]

Angiogenesis occurs after the development of a primary vascular plexus and can take two forms, sprouting and nonsprouting.[13] Sprouting angiogenesis refers to the development of new blood vessels through proteolytic degradation of the extracellular matrix, migration/proliferation of endothelial cells, lumen formation, and maturation of endothelial cells to functional capillaries. Nonsprouting angiogenesis, however, occurs by intussuception.[19] Here, there is splitting of primary vessels by transcapillary pillars. Endothelial cells proliferate within a vessel, resulting in an enlarged lumen that can be split by these pillars or alternatively, by fusion and splitting of capillaries. Sprouting angiogenesis occurs during yolk sac and embryonic development but can also occur in later organogenesis such as during brain development.[13] During embryonic development, this process is seen during heart and lung organogenesis. Nonsprouting angiogenesis takes place during lung and heart development but

predominates during the former because of the presence of endothelial precursor angioblasts that are critical to the vascularization of this organ.[20]

The molecular factors involved in sprouting and nonsprouting angiogenesis are not well understood. However, it is known that in knockout mice lacking the tyrosine kinase receptor TIE-2, there is a lack of capillary sprouting in the brain leading to embryonic death at day 10.[21] TIE-2 may be modulating growth factors such as VEGF in promoting sprouts of capillaries.[18,21,22] The differences between vasculogenesis and angiogenesis are also suggested by this receptor as its activity is not required for the former process.[23]

As the vasculature continues to develop and mature, the blood vessels are pruned so that the plexus consists of large and small vessels.[24] During this process, excess endothelial cells may be lost. Endothelial cells may also reassemble into other vessels or dedifferentiate. The molecular mechanisms that regulate these processes are unknown but may be related to downregulation of survival factors for endothelial cells such as VEGF.[25] Several other factors have been identified which may also be crucial to the maturation of blood vessels. These include Ang 1 and ephrin-B2, the former of which may be important for stabilizing vessel walls.[26] Ang 1 is an agonist for the TIE-2 receptor. Mouse embryos that lack Ang 1 or TIE-2 develop a primary vasculature; however, this vasculature fails to undergo further remodeling.[26] Ang 1 may be promoting interactions between endothelial cells and the surrounding pericytes and matrix. These support structures result in maturation of immature vessels.[27]

Another factor critical to remodeling is Ang 2. This factor also binds the TIE-2 receptor but acts as an antagonist.[28] Overexpression of Ang 2 in mice produced defects resembling those of Ang 1 or TIE-2 knockouts. Ang 2 expression was induced in endothelium undergoing remodeling especially during regression of vessels in ovaries or tumors.[9,28-32] This data suggested that Ang 2 was providing a destabilization signal in the initial phases of remodeling. During this destabilization, endothelial cells could be more sensitive to growth factors that are sequestered in the extracellular matrix. This might lead to sprouting angiogenesis. However, if growth factors are not present or are present at low levels, the vessels might undergo regression such as that seen during ovarian follicular regression.[12,26]

Another family of tyrosine kinase growth factor receptors are the ephrins, and these, along with their ligands, may also play a role in remodeling of the vasculature.[33-35] Knockouts of ephrin-B2 and its EphB4 receptor have defects in vascular development similar to Ang 1 and TIE-2.[33-35] Interestingly, ephrin-B2 is expressed on arterial vessels, whereas EphB4 is found on the venous side. Moreover, ephrin-B2 may be expressed on arterial endothelium, smooth muscle, and pericytes.[26] This factor may promote interaction of endothelial cells with smooth muscle during arterial development. Thus, these factors may be critical to differentiation and identity of vessels. Furthermore, the interaction of ephrin-B2 with its receptor Eph-B4 may be involved in the fusion of arterial and venous vessels.[33-35] During angiogenesis associated with the female reproductive system or tumors, ephrin-B2 is expressed on the vasculature, suggesting that sprouting of new vessels in these settings may arise from the arterial side as opposed to the existing theory that new vessels arise from postcapillary venules.[26] This data supports the role of ephrins and their receptors not only during vascular development but also during adult angiogenesis.

MECHANISMS AND GENETIC EVIDENCE FOR TUMORAL ANGIOGENESIS

The regulation of angiogenesis during tumor growth is becoming better understood. Several years ago, the balance hypothesis for the angiogenic switch was postulated.[36] The switch occurs during proliferation of endothelial cells as they organize to form new blood vessels. What determines whether the switch is on or off are the levels of angiogenic inhibitors compared with activators. More than 20 endogenous activators and inhibitors each have been characterized (Table 1). Whether a tumor remains in a dormant state versus it progressing may reflect the balance of these activators and inhibitors.[36] These factors may be regulated through changes in the microenvironment, emphasizing the importance of tumor cell-extracellular matrix interactions.

There are at least four potential mechanisms by which tumors can stimulate angiogenesis (Fig 2). The first hypothesis, put forth by Judah Folkman three decades ago, suggested that tumors cannot grow beyond 1 or 2 mm before stimulating the sprouting of new blood vessels.[2] Such a process involves the secretion by tumors of angiogenic growth factors which may stimulate tyrosine kinase activity in endothelial cells.[12] Tumors may secrete VEGF, and as they get larger, other angiogenic growth factors may be generated.[37] The second mechanism suggests that tumors can coopt existing vasculature.[9] Moreover, tumors grow around blood vessels and mediate vessel regression by upregulation of Ang 2. The secretion of this factor induces loosening of matrix structures and loss of vessel integrity. This results in central tumor necrosis. Hypoxia stimulates VEGF production and angiogenesis occurs around the periphery of the tumor.[9,38-40] Third, the regulation of angiogenesis may, in part, be contributed to by circulating hematopoietic precursors.[41-45] Circulating CD34+ endothelial cell precursors have been found in areas of angiogenesis in wounds and tumors.[46-48] The functional nature of these endothelial cells and their contribution to angiogenesis remains

Table 1. Endogenous Pro- and Antiangiogenic Agents

Stimulators	Inhibitors
VEGF	Interferons α/β
bFGF/aFGF	Angiostatin
PlGF	Endostatin
PDGF	Vasostatin
TGF α/β	Canstatin
Del-1	Tumstatin
TNF-α	VEGI
IL-8	Platelet factor-4
HGF	Thrombospondin-1
PD-ECGF	IL-12
Angiogenin	16-kd prolactin fragment
IL-3	PEDF
Proliferin	2-methoxyestradiol
Pleiotrophin	53-kd antithrombin III
Follistatin	Prothrombin fragments 1 and 2
Midkine	Domain 5 of HMWK
Leptin	Restin
G-CSF	Maspin
HIV Tat	SPARC
	IL-18
	IP-10

NOTE. Adapted from the Angiogenesis Foundation Clinical Trials Database (http://www.angio.org).

Abbreviations: FGF, fibroblast growth factor; PlGF, placental growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; Del-1, developmental endothelial locus-1; TNF-α, tumor necrosis factor alfa; VEGI, vascular endothelial growth inhibitor; IL, interleukin; HGF, hepatocyte growth factor; PD-ECGF, platelet-derived endothelial cell growth factor; PEDF, pigment epithelium-derived factor; HMWK, high molecular weight kininigen; G-CSF, granulocyte colony-stimulating factor; HIV Tat, human immunodeficiency virus TAT; IP-10, interferon-inducible protein-10; SPARC, secreted protein acidic and rich in cysteine.

undetermined and a subject of intense investigation. Evidence for genetic regulation of angiogenesis stems from studies using Id knockout mice. These are a family of developmental genes thought to be required for normal angiogenesis in mouse forebrain. Mice with deletion of one allele of *Id1* and two alleles of *Id3* have diminished or absence of tumor growth.[49] However, transplantation of mice with wild-type bone marrow can restore angiogenesis and rapid tumor growth.[48,50] This data further supports circulating hemangioendothelial precursor cells as contributing to angiogenesis, but to what extent this occurs in the adult host is yet to be described. It also remains to be seen whether there is redundancy in this regulation and if tumors can escape this inhibition over time. The fourth potential mechanism is known as vascular mimicry and will be discussed separately.

The role of angiogenesis in leukemic growth is also being studied as a potential critical step in development and progression of leukemia.[51] The increased microvessel density pattern in the bone marrow of children with acute lymphocytic leukemia (ALL) has previously been observed,[51] and now, this has been seen as well with acute myeloid leukemia.[52] The interaction of endothelial cells with leukemia cells may be significant with regard to growth factors secreted by each of these compartments that stimulate proliferation of both cell types. Leukemia cells may express VEGF and VEGF receptors such that there may be autocrine and paracrine loops regulating growth.[53,54] There is also evidence to suggest that highly proliferative blasts in ALL may become independent of stromal or angiogenic influences.[55] Angiogenesis inhibitors have shown activity in preclinical models for leukemia,[56] and some of these agents are being evaluated in patients with myelodysplastic syndrome and leukemia.[57,58] Whether these agents in vivo act directly on endothelial cells, leukemic cells, or both has yet to be elucidated.

VASCULAR MIMICRY

A number of studies have been undertaken to understand the complexity of the vasculature within a tumor as compared with normal tissues or wounds. These studies have used various scanning methodologies to evaluate vessel size, pore size, topographic structure, and vascular permeability. McDonald and Foss[5] have shown variability with endothelial pore size in tumors compared with normal tissues and have found irregularity and heterogeneity within the vessel. Studies on permeability using various dye reagents showed increased permeability in tumor vessels compared with normal vessels. Hendrix et al[59-63] and others have entertained the idea that in certain tumors, particularly uveal melanoma, tumor cells can form blood vessels and contribute to the functional vasculature themselves (vascular mimicry). If this is the case, then these tumor cells can be analyzed by subtractive hybridization/microarray methods to determine expression patterns that are similar to endothelial cells, particularly the blood vessel forming properties. Others have disputed this and reported that endothelial cells comprise the bulk of the vessel wall, whereas only a small percentage of the blood vessel wall is lined by tumor cells (3%).[5] There seems to be a dynamic interplay within angiogenic vessels with endothelial cells proliferating locally, tumor cells lining vessels and extravasating into the circulation, and possibly endothelial precursor cells contributing to new vessel formation.[50]

Recent data suggests that some tumor cell lines may express angiogenic growth factors and receptors. Masood et al[64] reported the expression of VEGF, Flk-1, and Flt-1 (VEGF-R1) in a variety of human tumor cell lines of nonendothelial origin. These included melanoma, ovarian, pancreatic, and prostate carcinomas. Antisense agents or antibodies directed against these factors led to inhibition of tumor growth in vitro. Based on this data, it was hypothesized that antiangiogenic agents

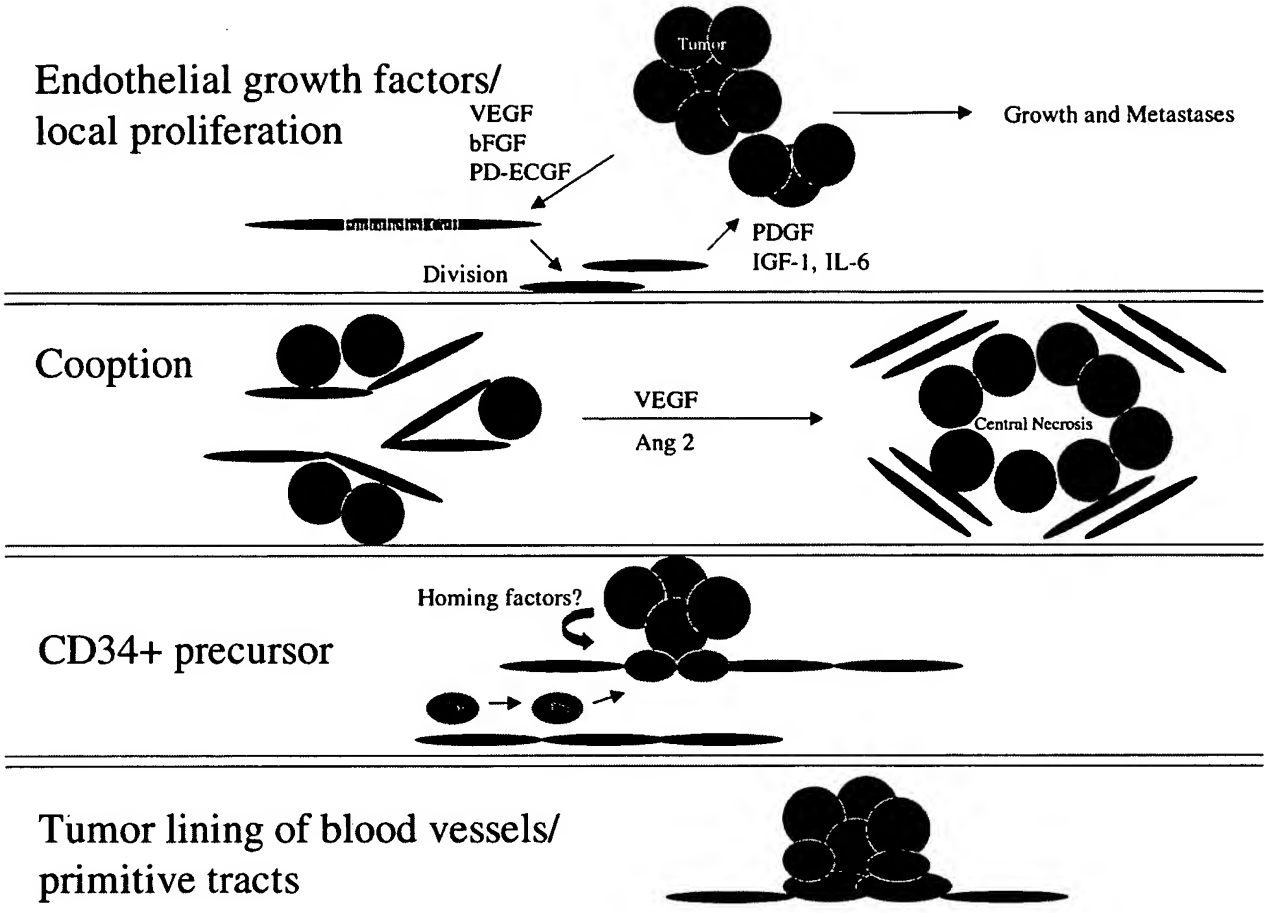

Fig 2. Shown are four potential mechanisms by which tumors may stimulate or contribute to angiogenesis.

may have a dual role in tumor growth inhibition in vivo (ie, inhibition of angiogenesis and direct cytotoxicity to tumor cells).[64] It may be the case then, that certain tumors such as melanomas may have primitive blood vessel–forming properties (considering their expression of angiogenic markers such as $\alpha_v\beta_3$ integrin, VEGF, Flk-1, and Flt-1, as well as preliminary structural evidence from the work of Hendrix et al[61,62] mentioned above).

DISCOVERY OF ENDOTHELIAL CELL TARGETS

Angiomics

To explore novel receptors on endothelial cells that are critical for signaling of growth, differentiation, or apoptosis, a variety of genetic screens have been devised. Oligonucleotide and cDNA microarray technologies are currently being used to characterize genes that are upregulated or downregulated in quiescent versus tumor endothelium.[65] In addition, serial analysis of gene expression methods have been used to evaluate differential gene expression.[6] Technologies such as these as they are applied to expression patterns in endothelial cells have been loosely termed angiomics.[66] The use of these technologies is helpful in describing new markers for targeting drugs and radioisotopes. However, they do not provide functional information on the role of candidate markers in stimulating growth or differentiation. Patterns of gene expression are useful in determining general properties such as the expression of adhesion molecules or matrix metalloproteinase enzymes in actively dividing versus quiescent endothelial cells. However, it remains difficult to determine which one or combination of candidate genes are important in the angiogenic phenotype. Another disadvantage of using microarrays to characterize interesting growth or inhibitor genes in angiogenesis is related to the discoveries that many potent endogeneous inhibitors of angiogenesis (ie, angiostatin, endostatin, and others) are fragments of larger precursor

molecules.[67,68] In other words, these inhibitors are cryptic and will not be represented by typical cDNA microarray chips. The use of proteomic approaches or protein lysate microarrays may overcome this obstacle. These methods are highly sensitive and quantitative. They also have the advantage of studying protein expression and phosphorylation status of signaling proteins. Furthermore, protein microarrays allow expression analysis of picogram amounts of protein from cell lines, tissues, or microdissected regions of tissue.[69] However, genetic screens with functional end points may be more insightful in determining molecules that may have potential in cancer therapy. The use of peptide or cDNA libraries to fish out interesting markers that confer survival of endothelial cells in response to an inhibitor may yield useful drugs for both stimulating and inhibiting angiogenesis.[70] Alternatively, the screen can be designed to select for molecules that upregulate growth factors or adhesion molecules (VEGF, intercellular adhesion molecule-1 [ICAM-1], platelet-endothelial cell adhesion molecule-1 [PECAM]) that are critical to angiogenesis.

Vascular Addresses

The normal vasculature varies from organ to organ within an animal.[71] Although there are many similarities, there are distinct addresses that can be used to develop peptides and drugs that target one vascular bed compared with another. The work of Arap et al[72] has demonstrated that phage libraries administered in vivo can be used to determine specific peptide sequences that allow phage to home to one organ versus another. Such peptides can be shown to be specific by repeated in vivo selection and rescue of phage. Many of the peptides that are home to tumors, for example, contain RGD and NGR sequence motifs.[73] The receptors for these as well as other homing peptides are being identified. For example, RGD-containing peptides bind to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, whereas peptides with NGR motifs bind to aminopeptidase N protein. Both the integrins and aminopeptidase N protein have been shown to be critical to angiogenesis because specific antibody and chemical inhibitors show inhibitory activity in mouse and chick angiogenesis models.[74,75] This in vivo phage display system represents a model to determine peptides that might be useful in targeting chemotherapeutic drugs, radiotoxic agents, biologic toxins, and thrombotic factors to tumors. Current efforts are underway to determine vascular addresses in patients.[76]

USE OF ANTIANGIOGENIC CHEMOTHERAPEUTIC DRUGS IN CANCER

Evidence has suggested that there are many chemotherapeutic drugs that have antiangiogenic activity. For a chemotherapeutic drug to be considered as having antiangiogenic activity, it must demonstrate this activity at a lower dose than would be required to kill tumor cells.[77] Some of these include cyclophosphamide,[78] paclitaxel,[79,80] doxorubicin,[81] and vincristine.[82] Several reports have indicated that scheduling chemotherapeutic agents with more frequent dosing can yield potent antiangiogenic activity in mouse tumor models.[78,83]

Delivery of antiangiogenic drugs in this fashion (ie, by continuous or frequent low-dose administration) has been termed metronomic dosing.[83-85] Because tumor-associated endothelial cells have lower rates of cell division than tumor cells themselves, such a strategy may potentially target the endothelial cell compartment. Bolus doses of chemotherapy given with prolonged rest periods may, theoretically, only weakly inhibit endothelial cell growth versus prolonged treatment schedules. Browder et al[78] showed that frequent, low doses of standard chemotherapeutic agents like cyclophosphamide may result in potent antiangiogenic activity compared with maximally tolerated bolus dosing. Endothelial apoptosis preceded apoptosis of tumor cells with appropriate antiangiogenic scheduling of these agents.[78] Although this antiangiogenic schedule did not completely eradicate tumors, it did result in a 2-month delay of tumor growth compared with standard dosing. Similarly, Klement et al[83] showed that low doses of vinblastine combined with anti–VEGF-R2 antibody in the treatment of human neuroblastoma xenografts in severe combined immunodeficiency mice resulted in regression of tumors without recurrence (provided that the treatments were continued).

In clinical practice, evidence for increased antitumor activity has been seen with weekly dosing of paclitaxel for treatment of breast cancer.[86] There is also evidence for increased response rates in patients with colorectal cancer receiving continuous-infusion fluorouracil (5-FU) versus bolus dosing in a recent meta-analysis.[87] Although efficacy measured in survival has been limited in these clinical trials, there are encouraging results from preclinical studies using combinations. Combining chemotherapeutic drugs like paclitaxel, for example, with antiangiogenic agents such as anti-VEGF monoclonal antibody may have synergistic effects on cell growth in preclinical models.[88] Further study of drug dosing levels and pharmacokinetics may yield information on the window of antiangiogenic activity of drugs that avoids toxic side effects on rapidly dividing tissue of the bone marrow and gastrointestinal tract.

Tyrosine kinase inhibitors, particularly drugs that target the epithelial growth factor (EGF) or erbB-2 receptors, may have antiangiogenic activity.[89-91] Moreover, EGF receptor inhibitors have been shown to suppress VEGF expression. Signaling through these receptors may regulate VEGF

expression and be a potential mechanism by which certain tyrosine kinase inhibitors inhibit angiogenesis in vivo. Inhibitors such as Herceptin (Genentech, South San Francisco, CA; directed against overexpressed EGF receptor family member erbB-2/Her2/*neu*), chimeric monoclonal antibody C225, and small molecule antagonists such as ZD1839 (directed against the erbB-1 receptor) may have antitumor activity as a result of antiangiogenic effects. In fact, resistance to these agents (for example, anti–EGF-R antibodies) has been shown to be related to increased VEGF expression and increased antiangiogenic potential in vitro and in vivo.[89] When considering these agents for clinical trials, it may be useful to combine them with agents that deplete VEGF, such as anti-VEGF antibodies, to significantly deplete circulating VEGF levels. In a similar way, they could be combined with drugs that block the VEGF-R2 receptor (ie, SU6668) to diminish ligand binding and receptor activities together. Alternatively, these anti–EGF-R agents could be used in combination with anti–bFGF-R inhibitors to potentially synergize in affecting two or more signaling pathways.

Thalidomide has resurfaced as a useful drug in cancer therapy. This drug achieved notoriety because of the birth defects that it caused in women receiving it for sedation during pregnancy. This agent has been shown to have pleiotropic effects including antiangiogenic, immunomodulatory, neurologic, and anti-inflammatory effects.[92,93] With regard to its antiangiogenic effects, it has been proposed to downregulate tumor necrosis factor alfa, bFGF, and VEGF and to show activity in vivo on the chick chorioallantoic membrane assay.[94] Singhal et al[95] have demonstrated the effectiveness of this drug in patients who have failed standard chemotherapy for multiple myeloma. Up to one third of patients in this setting showed a response to therapy. Thalidomide is also being evaluated in myelodysplastic syndrome, acute leukemia, AIDS Kaposi's sarcoma, glioblastomas, and others.[94,96-98] Demonstration of antiangiogenic effects in patients has been difficult, in part, related to the difficulty of reliable assays but may also be because of the pleiotropic effects of thalidomide.[95]

Cyclooxygenase inhibitors have evolved as new agents with a potential chemopreventative role in colorectal cancer.[99,100] The mechanism for the anticancer role is unknown but may be due to proapoptotic effects, antiproliferative effects, inhibition of cellular transformation/carcinogen activation, or immune modulation directed against the cancer cell.[101] These inhibitors may also have antiangiogenic effects, and the mechanism is unknown but could be related to the impaired production of prostaglandins as a result of cyclooxygenase inhibition.[102] Nonsteroidal anti-inflammatory drugs are the most commonly used cyclooxygenase inhibitors; however, these have nonselective effects. They inhibit both cyclooxygenase 1 and 2 enzymes (COX-1 and COX-2, respectively) and may impair the angiogenesis associated with wound healing.[103] Novel specific inhibitors have been developed against COX-2 that may avoid the gastrointestinal side effects associated with COX-1 (ex, celecoxib and rofecoxib). Some of these COX-2 inhibitors may prove beneficial in prevention of colonic polyps and carcinomas and possibly in treatment of established tumors.

EXPERIMENTAL THERAPIES

Endogenous Antiangiogenic Factors

Along with the many endothelial cell growth factors (VEGF, bFGF, placental growth factor [PlGF], transforming growth factor alfa [TGFα], and others), numerous endogenous antiangiogenic proteins have been characterized (Table 1). It is becoming more evident that many of these factors are fragments of larger precursor molecules[67,68,104,105] some of which mediate coagulation and fibrinolysis.[106] Some of these angiogenesis inhibitory factors include domain 5 of HMWK,[104] prothrombin fragments 1 and 2,[105] and AT-III.[107] In addition, regulators of angiogenesis in the fibrinolytic pathway include α2-antiplasmin,[108,109] α2-macroglobulin,[109,110] plasminogen activator inhibitor,[109,111] and possibly angiostatin.[67,112] As tumors or wounds disrupt basement membranes, new cryptic polypeptides with antiangiogenic activity may be exposed. These may come from collagen, fibronectin, and other matrix components. Degradation components with biologic activity may be generated by matrix metalloproteinases and other enzymes secreted by tumors and other cell types.[113] Thus, the hemostatic system is likely an important regulator of angiogenesis, particularly since angiogenesis is a step in the repair of wounds. Perhaps the dysregulation of tumor growth and the disruption of the tight balance between endothelial growth and inhibitory signals distinguishes tumoral angiogenesis from wounds that heal.[114,115]

Antiangiogenic Gene Therapy

The use of gene therapy for delivery of antiangiogenesis genes has shown promise in preclinical models in mice but has not yet been tested in patients.[116-118] Because the goal of antiangiogenic therapy of cancer is long-term suppression of neovascularization and tumor growth, delivery and prolonged expression of genes in the host is the ideal strategy.[119] It also represents a method for avoiding the problems seen with delivery of many recombinant proteins including their stability and solubility.[120] A variety of viral and nonviral methods are being assessed; these include retroviruses/lentiviruses,[56,121-124] adenoviruses,[125-127] adeno-asso-

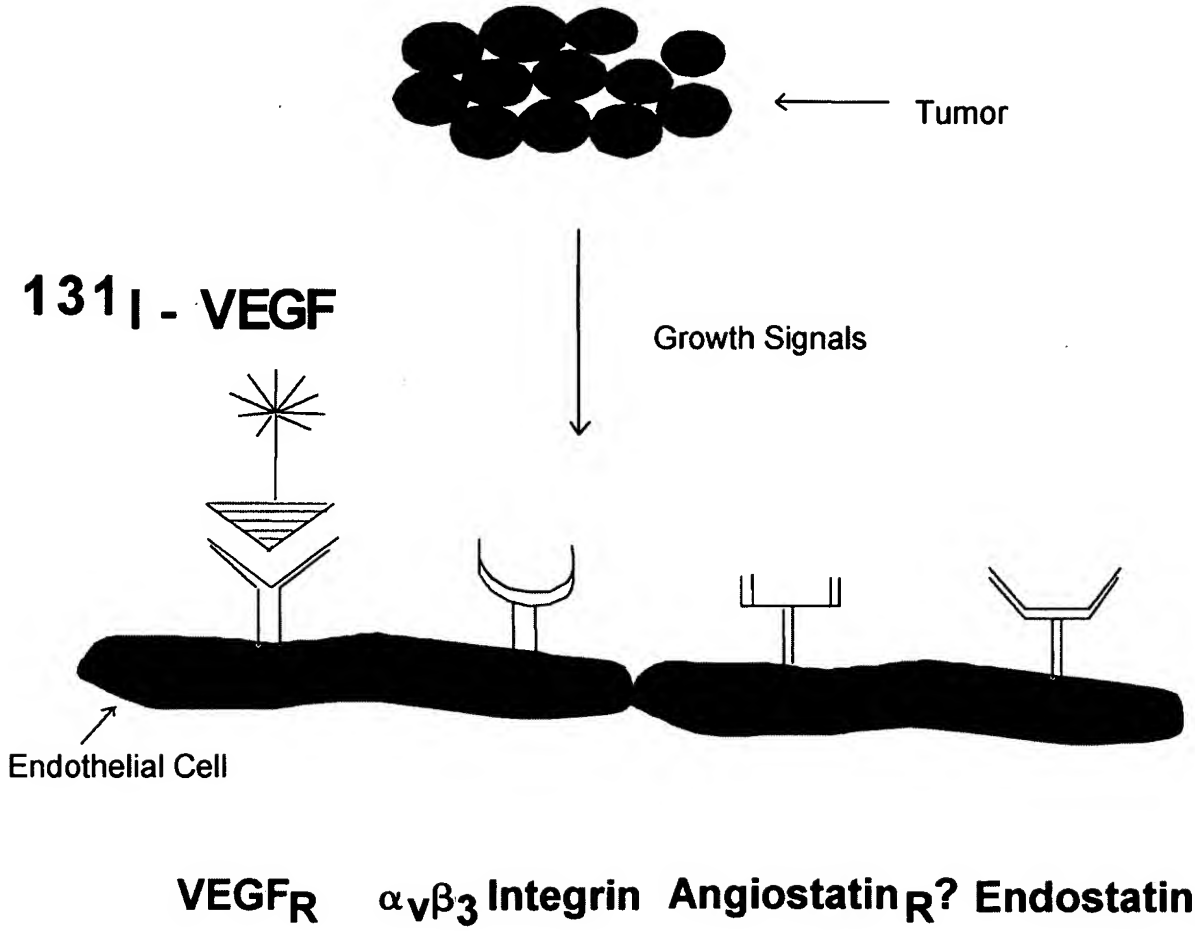

Fig 3. Development of antiangiogenesis radioligands. Shown here is a potential way to use tumor vasculature for novel imaging strategies and the development of a novel angiogenic-specific radiotherapy. Tumor vasculature-specific angiogenic or antiangiogenic ligands are conjugated to radionuclides and administered to the host followed by SPECT or PET imaging/therapeutic effect.

ciated viruses,[128] cationic liposomes/microencapsulation,[129-132] and naked DNA delivery.[133] The use of viral vectors that can integrate DNA into the genome offers advantages for long-term gene expression compared with transient expression of genes, which may be similar to delivery of bolus protein. On this basis, the use of lentiviruses and adeno-associated viruses might prove advantageous, especially when planning for delivery into nondividing cells such as muscle and liver. Delivery of these genes and maintenance of long-term gene expression at high levels remains challenging in vivo. Delivery of antiangiogenic genes to glioblastoma multiforme tumors may, in theory, be ideal for this form of therapy.[120,134] Because this type of tumor rarely metastasizes outside of the CNS, then high systemic levels of gene expression may not be necessary. Delivery of antiangiogenic genes could be targeted to residual brain tumor by stereotactic injection (such as by delivery with retroviral packaging cells).[135,136] It may be that antiangiogenic gene therapy could complement existing standard therapies and may result in synergistic interactions with chemotherapy, radiation, and other biologic therapies (ie, immunotherapy and antiangiogenic drugs).

Antiangiogenic Radioligands for Cancer Imaging and Therapy

Targeting the vasculature may be useful in new strategies for both imaging of tumors and treatment (Fig 3). Antibodies, peptides, and other small molecules carrying isotopes with imaging properties can be administered during tumoral angiogenesis.[137-140] Potential examples of these include radiolabeled VEGF as well as anti-VEGF receptor antibodies. This approach has already been used with $\alpha_v\beta_3$ integrin-binding peptides, antithrombomodulin antibodies, and antiendoglin antibodies with promising preclin-

ical results.[138,141-143] For example, investigators have used paramagnetic liposomes covalently attached to anti-$\alpha_v\beta_3$ antibodies to demonstrate targeted delivery of a magnetic resonance contrast agent to image tumor vasculature in VX2 rabbit carcinoma.[139] In treatment models, Tabata et al[143] have shown that ^{125}I-labeled antiendoglin monoclonal antibodies significantly inhibited human breast cancer tumor growth in severe combined immunodeficiency mice compared with ^{125}I-labeled antibody controls that showed no effect.

Theoretical advantages for development of antiangiogenic radioligands are multiple. First, it may be possible to detect a tumor at an early stage of growth (ie, at the 1 to 2 mm stage when the angiogenic switch is turned on). Second, because the receptor-ligand interaction is intravascular, then theoretically the imaging should be rapid barring unusually slow clearance of the reagent from the circulation. This would not require tumor cell uptake or metabolism, which is necessary for many of the current contrast reagents. Third, the imaging data would provide functional insight and would depict areas of angiogenesis within a mass. This could be helpful in distinguishing a benign mass from a tumor undergoing rapid growth and neovascularization. Finally, this strategy may be applicable to imaging many tumor types, especially if the endothelial cell target chosen is common to the vascular bed of tumors and does not vary regardless of the organ that the tumor is growing. With regard to therapy, there are several advantages for using a radioactive tag on a vasculomimetic drug. One of the major obstacles to radioimmunotherapy of tumors targeting tumor antigens such as CD20 and others relates to the difficulty penetrating a large bulky mass.[144,145] High interstitial pressures and poor lymphatic drainage may make certain areas of the tumor inaccessible to treatment. However, targeting the vasculature provides easy access with potentially a large bystander effect (ie, one endothelial cell may support 50 to 100 tumor cells).[2] There may be a dual mechanism by which antiangiogenic radioligands may inhibit tumors. The intrinsic antiangiogenic activity of the ligand coupled with the radiation emitted may synergize in killing endothelial cells and thereby indirectly killing tumor cells. The other mechanism is via a direct radiotoxic effect on tumor cells. This depends on the path length of the particulate energy such as the β particle emitted from many isotopes like I-131 and Cu-64. It also depends on the intrinsic radiosensitivity of the tumor cell. This approach may be independent of tumor cell type and growth fraction.

Endothelial Cell-Based Therapies

Several reports have indicated the presence of a circulating bone-marrow derived population of CD34+ endothelial cell precursors.[41-44,48] These precursors have been found to line vascular grafts, prostheses, and newly formed blood vessels during tumor growth.[47,146-150] The percentage of tumoral vessels that are lined by CD34+ derived precursors from bone marrow can be as high as 90% from one report.[48] The isolation of CD34+ stem cells from bone marrow followed by genetic manipulation may be exploited to deliver suicide genes or antiangiogenic factors to shrink tumors. A recent study using herpes simplex virus thymidine kinase transduced CD34+ cells administered intravenously to primates undergoing skin grafting has shown rejection of the graft after administration of gancyclovir.[46] This study suggests a role for these precursor cells in contributing to neoangiogenesis. The significance of passive transfer of these precursors or of endothelial cells at different stages of differentiation in contributing a functional role to the vasculature remains to be determined.[151]

Endothelial Cell Vaccines and Immunotherapy

Active and passive-based immunotherapies of cancer directed at endothelial cells remain experimental, although very encouraging. There are several antibodies already in clinical use that target various angiogenic factors and their receptors. For example, anti-VEGF antibody has completed phase I trials in colorectal and lung cancers with encouraging data on responses.[152] Monoclonal antibodies directed against VEGF-R2 receptor or endoglin are also in clinical trial.[153] Similarly, small molecule drugs and antibodies targeting $\alpha_v\beta_3$ integrins on endothelial cells are being studied as a strategy for inducing apoptosis of tumor-derived endothelial cells. These monoclonal antibodies may be useful alone or in combination to stabilize the growth of tumors and prolong disease-free survival.

Endothelial cells have also been evaluated in vivo as a source of immunogens that could potentially induce host responses against angiogenic endothelial cells within a tumor. Wei et al[154] have shown that primary xenogeneic endothelial cells can be administered to mice with subsequent immunoprotective effects on tumor growth and therapy of established tumors. There was demonstrable inhibition of angiogenesis in vivo and that the arm of the immune response critical for the effect was humoral based on depletion studies of CD4+ and CD8+ T cells. Passive transfer of serum antibodies from vaccinated mice to naïve mice showed protection after tumor challenge in these mice. Furthermore, the antibodies did not cross-react with tumor cells supporting the idea that the vasculature was the main target of the immune response. No gross toxicities were observed by these investigators. The use of xenogeneic endothelial antigens resulted in breaking of immune tolerance to self antigens on proliferating endothelium. This

approach has demonstrated that antiangiogenic immune responses are independent of tumor type because endothelial cell-vaccinated mice were protected against a variety of syngeneic tumors. Similarly, administration of a liposome-encapsulated FGF-2 peptide vaccine demonstrated antiangiogenic immune responses and prevented tumor growth in a preclinical model.[155] We have also observed a decrease in basal VEGF levels in endothelial cell-vaccinated mice, suggesting a protective antiangiogenic phenotype. We have further confirmed protection against melanoma tumor challenge after vaccination with syngeneic transformed endothelial cells (data unpublished). Therefore, directing an immune response against active and growing endothelial cells may potentially provide a pan-curative or pan-protective vaccine that could inhibit the growth of any tumor type.

Matrix Metalloproteinases (MMP)

MMPs are a family of zinc-dependent proteinases that mediate degradation of extracellular matrix which is important for tumor cell invasion and metastasis.[156,157] These enzymes have been classified into various groups depending on their substrates (ie, collagenases, gelatinases, stromelysins, membrane-type, and nonclassified).[158] Although these enzymes are absent or expressed at very low levels in normal tissues of the host, they have been found to be upregulated in malignant tissues. It is becoming increasingly clear that most MMPs are made by stromal cells versus carcinoma cells. Cancer cells may be inducing stromal cells to produce MMPs by extracellular matrix metalloproteinase inducer and cytokine stimulatory mechanisms.[159] These MMPs are known to be regulated at the level of transcription, proteolytic activation of the precursor zymogen, and inhibition of the active enzyme by tissue inhibitors of MMP.[156] Inhibition of MMPs, such as MMP-9, has shown activity against metastases in a rat sarcoma model.[160] In another report, MMP inhibitors (MMPIs) were shown to induce Fas-mediated apoptosis of Ewing's sarcoma cell lines by inhibiting the release of soluble Fas ligand (FasL) from the transmembrane surface (Ewing's sarcoma family of tumors are known to express functional transmembrane FasL). It was hypothesized that accumulation of FasL on the membrane surface promoted apoptosis of the cells.[161] Some MMPs, such as MMP-2, may have effects on early stages of angiogenesis when the breakdown of basement membranes is required. Other MMPs may act later in tumor growth and potentially generate angiostatin from plasminogen or release of growth factors from the extracellular matrix.

Numerous MMPIs are being evaluated for stromal therapy in a variety of cancers and are at various stages of clinical development. Some of these agents, including BMS-275291, Neovastat (AEterna Laboratories, Quebec, Canada), and Col-3, are presently undergoing evaluation in human trials and have shown promise in preclinical studies.[162-168] Many trials have incorporated MMPIs in prevention studies and evidence for reduction of recurrence rates will take large randomized trials at multiple institutions over many years.[169] In particular, these agents are being studied in the adjuvant as well as advanced settings for a variety of sarcomas and other solid tumors.[170] As such, a certain MMP (ie, MMP-7) has been implicated in early stages of tumor growth; therefore, inhibitors of this particular MMP may have a role in primary or secondary chemoprevention strategies for tumors.[171]

The most prominent toxicity seen with these MMPIs is the development of inflammatory polyarthritis.[172] Many of the above inhibitors are nonspecific and act on numerous MMPs. Some of theses MMPs, such as MMP-1, may be implicated in the musculoskeletal side effects.[173] The mechanism for this toxicity may be related to the inhibition of membrane receptor shedding by MMPIs such as the extracellular domain of the tumor necrosis factor receptor.[157,174,175] Newer inhibitors are being studied that lack this antisheddase activity.[157] The challenge will be to develop MMPIs that are specific to only one of the MMPs and will result in elimination of unwarranted toxicity.

Although these inhibitors have antiangiogenic and antimetastatic effects, resistance to therapy could develop. Evidence suggests that tumor blood vessels could produce MMPs with reduced affinity to MMPIs and that suppression of these enzymes by MMPIs could result in the production of other matrix degrading enzymes.[176,177] The challenge for stromal therapy of cancer will be to produce MMPIs or a combination of these that can be used to inhibit specific subtypes of MMPs involved in tumor invasion and metastasis without generating toxicity and resistance.[169]

Combination Treatments

A number of studies have indicated advantages for combining antiangiogenic agents with each other,[56] as well as with chemotherapy[88] and radiation.[178] The antiangiogenic and antitumor effects have been shown to be additive and synergistic.[56,179,180] Combining multiple agents that target a number of endothelial cell pathways may yield potent proapoptotic and growth inhibitory effects. For example, one study has shown that delivery of a combination of genes encoding the antiangiogenic proteins, angiostatin and endostatin, can yield inhibitory effects on tumor growth in mice bearing B16F10 melanoma and L1210 leukemia.[56] In other reports, administration of recombinant proteins together or with chemotherapeutic drugs yielded potent antitumor effects in an ovarian carcinoma model and a spontaneous pancreatic adenocarcinoma model

using RIPTag transgenic mice.[179,180] The mechanism for chemotherapeutic + antiangiogenic combination effects may be related to increased access of chemotherapeutic drugs as a result of permeability from antiangiogenic effects on endothelial cells. In patients harboring extremity sarcomas, the combination of tumor necrosis factor alfa with high-dose melphalan has been found to regress large established tumors of the extremity by isolated limb perfusion.[181] Other possibilities include synergistic proapoptotic effects on endothelial cells in combination with direct effects on tumor cells. With regard to the combination of radiotherapy and antiangiogenic treatments (particularly anti-VEGF antibodies), the radiation may be inducing damage to tumor cells within the center of a tumor via oxygen radicals.[9] Hypoxic tumor cells are known to upregulate hypoxia inducible factor and, subsequently, generate increased expression of VEGF.[38-40] The combination of radiation and anti-VEGF therapy has been shown to have potent antitumor effects along with decreased resistance of tumor cells.[182] Thus, the combination may be blocking the ability of tumors to escape by inhibiting VEGF and subsequent new blood vessel growth.[182]

Another approach taken to achieve the antiangiogenic potency of a combination of biologic agents is to fuse the agents together.[183] Using molecular cloning techniques, Scappaticci et al[183] created an angiostatin-endostatin fusion protein (Statin-AE) and demonstrated enhanced antiangiogenic and antitumor activity in vivo compared with either agent alone. This activity mirrored or improved the effects of combining angiostatin and endostatin proteins together. Although the mechanism for this activity is at present unknown, it may be that two receptors are crosslinked, the conformation of the chimeric protein has favored enhanced binding or activity to a single receptor, or that the fused protein shows greater stability than either protein alone.[183] These results suggest potential benefit in fusing two known potent antiangiogenic proteins and might be an approach for creating novel antivascular drugs for cancer therapy.

CLINICAL TRIALS WITH ANTIANGIOGENIC AGENTS

What Are We Learning About Antiangiogenic Agents in Clinical Trials?

There are over 75 antiangiogenic agents in clinical trial. Most of these are in phase I or II trials. At least 12 of these agents have entered or completed phase III (Table 2).[94,99,100,152,157,167,168,184-206] There are no currently Food and Drug Administration–approved antiangiogenic agents for cancer therapy; however, agents have been approved for some physiologic/pathophysiologic conditions such as PDGF for wound healing and Visudyne (Novartis, Basel, Switzerland) for macular degeneration. The theoretical advantages that antiangiogenic agents may have in treatment of cancer are several-fold. The agents may have easy access to tumoral endothelial cells compared with drugs that act on tumor cells directly and have to penetrate large bulky masses. Antiangiogenic drugs may not cause cytopenias and thus will avoid many of the unwarranted toxicities of standard chemotherapeutic agents. Theoretically, they might also avoid tumor resistance mechanisms if they are directly cytotoxic to endothelial cells. If antiangiogenic agents are successful, they might be applicable to many tumor types and not be dependent on cell type or growth fraction of cells within a tumor.

However, there are several main obstacles with regard to using antiangiogenic drugs in clinical trials. These include the following: (1) determining the appropriate dose from phase I trials to proceed to subsequent trials, (2) scheduling of drugs, (3) biological correlates, (4) appropriate use of these agents in clinical settings, (5) how best to combine these treatments with chemotherapy, radiation, or other biological therapies, and (6) necessity for angiogenic profiling (customized therapy). To overcome these obstacles will take further preclinical experimentation as well as insight from early clinical trials with antiangiogenic agents. Drugs such as SU5416, which showed promise in animal models as well as early clinical trials, have been shelved because of a lack of efficacy in phase III trials. Similarly, some of the MMPIs in phase III trials; namely, marimastat, prinomastat (AG3340), and BAY 12-9566 have shown no clinical efficacy.[159] In fact, the latter two drugs have been pulled out of further clinical trials by Agouron and Bayer, respectively. Patients receiving BAY 12-9566 in a small-cell lung cancer trial had worse outcomes regarding response rates than patients not receiving the drug. How these MMPIs affect angiogenesis in man in unknown. It may be that MMPIs are affecting cancer at early stages and not when the disease has progressed to advanced stages. Alternatively, perhaps immune-mediated effects on tumor cells are inhibited by MMPIs because of inability of immune cells to invade tumors and destroy them. In the future, design of clinical trials for MMPIs should incorporate patients with early-stage cancer, enroll only those patients who have high expression of MMPs, use a biological correlate for activity of MMPIs in vivo, and combine these agents with chemotherapy. Therefore, a better understanding of these concepts is desperately needed.

Dose-finding of antiangiogenic agents. Because these agents do not result in the usual toxicities seen with chemotherapy agents (ie, bone marrow or gastrointestinal tract), the appropriate dose that confers optimal antiangiogenic activity may be difficult to determine. A dose-limiting

Table 2. Angiogenesis Inhibitors in Clinical Trial:

Drug	Mechanism	Trial	Sponsor	Reference No.
Direct acting inhibitors of endothelial cells/receptor antagonists				
Thalidomide	Decrease TNFα, bFGF, VEGF	Phase I malignant glioma, phase I/II for advanced melanoma, phase II ovarian, metastatic prostate, phase II with chemotherapy against solid tumors, adjuvant study in recurrent or metastatic colorectal cancer; myelofibrosis with myeloid metaplasia, follicular lymphoma myelodysplastic syndrome, refractory ovarian, phase II gynecologic sarcomas, liver cancer; metastatic melanoma, CLL, multiple myeloma; phase III non–small-cell lung, nonmetastatic prostate, refractory multiple myeloma, renal cancer	Celgene, Warren, NJ	94
SU6668	Blocks VEGF-R2, FGF-R PDGF-R	Phase I for advanced tumors	Sugen, South San Francisco, CA	187, 188
Squalamine	Inhibits sodium-hydrogen exchanger (NHE3)	Phase II non–small-cell lung cancer; phase II ovarian; brain; phase I advanced cancers	Genaera Corp, Plymouth Meeting, PA	200
ZD1839	EGF-R inhibitor	Phase III non–small-cell lung cancer	AstraZeneca, London, UK	184
Erbitux (C225)	Monoclonal antibody against EGF-R	Phase II/III advanced solid tumors	ImClone, New York, NY	185
IMC-1C11	VEGF-R2 inhibitor	Phase I metastatic colorectal	ImClone	189
Angiozyme	Inhibits VEGF-R2 and -R1	Phase II breast and colorectal cancer	Ribozyme Pharmaceuticals, Boulder, CO	190
Endostatin	Glypicans, tropomyosin, $\alpha_v\beta_3$ integrin, MMP	Phase II neuroendocrine tumors and metastatic melanoma	EntreMed, Rockville, MD	192-195
Angiostatin	ATP synthase, Angiomotin, $\alpha_v\beta_3$	Phase I advanced tumors	EntreMed	196-198
Indirect acting/growth factor inhibitors				
Rhu Mab VEGF	Monoclonal antibody against VEGF	Advanced head and neck. Phase II metastatic renal cell cancer, phase II with chemotherapy in untreated advanced colorectal, metastatic breast; phase II non-Hodgkin's lymphoma, hematologic malignancies, metastatic prostate, previously untreated advanced colorectal, inflammatory breast cancer, advanced or recurrent cervical, non–small-cell lung; phase II/III advanced non–small-cell lung; phase III with chemotherapy in untreated metastatic colorectal, phase III metastatic breast	Genentech	152
MMPI				
BMS-275291	Synthetic MMP inhibitor	Phase I/II Kaposi's; phase II/III advanced or metastatic non–small-cell lung	Bristol-Myers Squibb, New York, NY	201
COL-3	MMP-2 and -9 inhibitor	Phase I/II brain, Kaposi's sarcoma	CollaGenex, Newtown, PA	204, 205
Neovastat	Natural MMP inhibitor	Phase II multiple myeloma, phase III renal cell cancer, phase III non–small-cell lung cancer	AEterna, Quebec, Canada	167, 168
Inhibitors of adhesion molecules/integrin signaling				
Vitaxin	Monoclonal antibody against $\alpha_v\beta_3$	Phase I/II trial in irinotecan-refractory advanced colorectal cancer	MedImmune, Gaithersburg, MD	191
EMD121974	Small molecule blocker of integrin (anti-$\alpha_v\beta_3$)	Phase I in patients with HIV-related Kaposi's sarcoma, phase III progressive or recurrent anaplastic glioma	Merck KGaA, Darmstadt, Germany	186

Table 2. (Continued)

Drug	Mechanism	Trial	Sponsor	Reference No.
Unknown or nonspecific mechanism of action				
Interferon α-2a	Decrease bFGF, VEGF	Advanced tumors (phase II/III)	Commercially available	186
Panzem (2-ME)	Unknown	Phase I/II solid tumor studies	EntreMed	199
Celecoxib	COX-2 inhibitor	Phase I prostate; phase I/II cervical; phase II basal cell, metastatic breast	Pharmacia, Peapack, NJ	99, 100
IL-12	Up-regulation of interferon γ and IP-10	Phase I/II Kaposi's sarcoma	Genetics Institute, Cambridge, MA	202
CAI	Inhibitor of calcium influx	Phase I studies in combination against solid tumors, phase II ovarian cancer, metastatic renal cell cancer	National Cancer Institute	203
IM862	Unknown	Phase II for untreated metastatic cancers of the colon and rectum; ovarian	Cytran, Kirkland, WA	206

NOTE. Adapted from National Cancer Institute Clinical Trials Database (*http://www.nci.nih.gov/clinical trials/doc.aspx?viewid=B0959CBB-3004-4160-A679-6DD204BEE68C*) and Kerbel.[66]

Abbreviations: ATP, adenosine triphosphate; CLL, chronic lymphatic leukemia; HIV, human immunodeficiency virus.

toxicity may not be reached with these agents. It is likely that the optimal biologic dose is not the maximally tolerated dose. For example, in a randomized phase II trial comparing two different doses of anti-VEGF monoclonal antibody in combination with fluorouracil/leucovorin treatment for metastatic colorectal cancer, a dose of 5 mg/kg showed higher response rates (42%) compared with a higher dose of 10 mg/kg (25%). There was also a prolonged time to progression in the lower dose arm compared with the higher dose when the study was reviewed in a blinded fashion by an independent review facility. The best way to determine appropriate biologic doses is to have reliable biologic correlates. Because these have yet to be optimally discerned, the dosing problem remains a challenge. Many clinical trials have been discontinued because tumors have not decreased in size by radiologic measurements. However, this approach may not be accurate because many of these agents induce disease stabilization. There is also evidence in preclinical studies with angiostatin and endostatin that the onset of the antiangiogenic effect may take days to weeks.[207,208] It may be reasonable to continue therapy for 3 months unless a biological correlate demonstrates that the drug has no antiangiogenic activity in vivo before this time period.

Scheduling of drugs. Because antiangiogenic therapy is considered to be long-term, chronic therapy for suppression of primary tumor growth and metastases, the optimal scheduling of these drugs needs to be determined. Some preclinical studies with endostatin have shown low-dose, continuous infusion to have more antitumor activity compared with bolus dosing.[209] However, many of these studies lack pharmacokinetic information. It is necessary to measure levels of the drug or its metabolites in the blood and to determine the best route and form of delivery based on chronic maintenance of effective therapeutic concentrations. This information may vary from patient to patient as well as from one disease state to another. Therefore, more pharmacokinetic data of new antiangiogenic drugs from preclinical studies is necessary on entry to clinical trials. Design of phase I trials should include measurement of drug levels in the blood because it is so critical for these agents to maintain a consistent therapeutic drug level for chronic suppression of angiogenesis and tumor growth. It may be akin to the maintenance of phenytoin levels as prophylaxis in patients with seizure disorders.

Biologic end points. The biologic end points for antiangiogenic therapy remain controversial. Determining the antiangiogenic activity of inhibitors in patients is challenging, in part, because tumor tissue may not be easily available for immunohistochemical and gene expression studies. Assessing microvessel density within tumors after treatment with an agent might be a theoretical approach for determining angiogenic status; however, there has been recent data suggesting that the microvessel density may increase, decrease, or stay the same after antiangiogenic therapy.[210] It may depend on the rate of apoptosis of the endothelial compartment versus the tumor cell compartment after antiangiogenic therapy, which will result in variable tumor cell-capillary distances. Alternatively, tumor cells may adapt and survive with low oxygen tension and can also assemble around remaining microvessels during antiangiogenic therapy resulting in variation in microvessel density. The use of angiogenic markers such as serum VEGF, bFGF, PDGF, and others may be less reliable and be reflective of tumor burden overall. Clinically, the use of VEGF to monitor prognosis of a variety of cancers has had

mixed results with the majority of studies showing some correlation but few studies showing none.[211] Some investigators are now studying endothelial cell shedding from tumor vasculature as a correlate for antiangiogenic activity of drugs. Methods are currently being developed to detect endothelial cells in the circulation of patients.[212] Other methods to determine angiogenesis in vivo include imaging strategies to evaluate perfusion, angiography, sonography, and magnetic resonance visualization of tumor vasculature.[213] The use of fluorodeoxyglucose and positron emission tomography (PET) imaging was used to monitor regional blood flow in tumors of patients participating in a recent phase I trial of endostatin.[214] Evaluation of tumor-free progression, time to progression, and disease stabilization may be the best way to assess new inhibitors in clinical trial.[11] Because most of these inhibitors may be blocking new angiogenic growth of tumors, regression of disease may be difficult to achieve. The goal of this type of treatment is to prevent any further growth of tumors.

Optimal clinical settings. How best to use antiangiogenic agents in cancer therapy is not known. Many phase I trials have entered patients with metastatic disease after failure of standard therapies. However, it may not be the best way to incorporate these drugs into clinical trials. To gain insight on the effectiveness of these agents on inhibition of angiogenesis in vivo, it may be best to incorporate them initially in patients who have been successfully treated and deemed free of disease (ie, the adjuvant setting). Patients who have a high likelihood of tumor recurrence after surgical removal, such as stage IIIa lung cancer or glioblastoma multiforme, should be considered as candidates for these trials. However, it may take many years with large numbers of patients at multiple centers to determine if progression-free or overall survival is impacted. The use of these drugs for metastatic disease could eventually be considered, especially if a drug has been shown to be effective in the adjuvant setting. The nature of the neovessels in advanced disease may be heterogeneous and more difficult to treat with antiangiogenic agents compared with microscopic or early-stage disease where there may be a more uniform nascent vasculature. This approach may prevent a useful antiangiogenic drug from being prematurely discarded especially if it does not show activity in advanced disease.

Incorporation of antiangiogenic agents with chemotherapy, radiation, and other biologic therapies. How to use these antiangiogenic agents with other modalities or other biologic agents has not been determined. Because some chemotherapeutic drugs have antiangiogenic activity, perhaps there may be synergistic antitumor effects. On the other hand, there may also be enhanced toxicity with regard to thromboembolic events.[215] These antiangiogenic agents may also enhance delivery of chemotherapeutic drugs to tumors. This may be related to pruning of the vasculature with antiangiogenic therapy, which could lead to normalization of the vasculature, decreased vessel density, decreased interstitial fluid pressure, and increased delivery of therapeutics.[216] With regard to combining angiogenesis inhibitors with radiation, perhaps they may work as radiation-sensitizers. In mouse models, Paris et al[217] showed that microvascular endothelial cells are a primary target of radiation damage. There is also evidence that radiation therapy delivered to tumors in mice can be enhanced if mice are treated with angiostatin.[218] Therefore, angiogenesis inhibitors could be used for enhancing the local effects of radiotherapy and perhaps leading to lower regional recurrence rates. The use of antiangiogenic agents can also be envisioned in combination therapies with biologic agents that have already been approved for cancer. Interferon alfa has been approved for the treatment of melanoma in the adjuvant setting as, for example, when lymph nodes are involved or in the advanced stages. Because interferon alfa may also have antiangiogenic activity, then combining it with thalidomide or anti-VEGF antibody might have synergistic activity. These combinations are being studied for renal cancer, and there are also some anecdotal reports of activity in sarcomas.

Angiogenic profiling (customized therapy). It has been shown that early in tumor development, one or several angiogenic factors are secreted by a tumor. With further progress of the tumor, there are other angiogenic factors that are added. For example, a human breast cancer tumor may express VEGF initially but may be expressing VEGF, bFGF, TGF β-1, PlGF, platelet-derived endothelial growth factor (PD-ECGF), and pleitrophin in its advanced stages.[219] With this knowledge, it may be envisioned that antiangiogenic therapy could be customized depending on the angiogenic profile of a patient's tumor and blood. Therefore, it may be that anti-VEGF monoclonal antibody could be effective in early stages, but that in advanced stages, it may be best to combine this antibody with a tyrosine kinase inhibitor that could interfere with signaling mediated by other growth factors. Genomic and proteomic arrays of tumor tissue could help identify a variety of highly expressed angiogenic factors and lead to customized therapy.

Toxicities

Potential toxicities of antiangiogenic agents must be considered as these agents are tested in patients.[119] Toxicities related to angiogenesis during normal processes of wound healing, ovulation, and pregnancy may be expected.[220,221] Similarly, ischemic diseases may be exacerbated

during treatment with angiogenesis inhibitors. Disruption of tumor endothelium could potentially lead to hemorrhage and set off a clotting cascade along denuded vessels. In trials of anti-VEGF antibodies in colorectal and lung cancers, thrombotic and bleeding events occurred, respectively.[152,222,223] Stroke, myocardial infarction, and pulmonary embolism are potential side effects and might be exacerbated in the setting of chemotherapy being administered concurrently with these inhibitors. For example, in a recent clinical trial of cisplatin, gemcitabine, and SU5416, eight out of 19 patients experienced thromboembolic events including transient ischemic attacks, cerebrovascular accidents, and deep vein thromboses.[215] Such a combination may have produced changes in the coagulation system along with changes in vascular wall integrity.[224]

The angiogenesis of wound healing may be fundamentally different than that associated with tumor growth. In mice, for example, angiostatin and endostatin have been shown to inhibit tumor angiogenesis with little or no effect on wound healing.[116] Direct evidence that certain angiogenesis inhibitors may have minimal effects on wound healing has come from studies evaluating tensile strengths of wounds.[221] Although endostatin appears not to grossly affect cutaneous wound healing in mice, there may be abnormalities in blood vessel maturation.[220] There are likely to be many similarities between normal and pathologic angiogenesis and potentially some of the inhibitors may affect both processes. Genetic studies evaluating gene expression patterns in endothelial cells in wounds versus tumors will provide insight on the subtle differences between these two angiogenic processes.

RESISTANCE TO ANTIANGIOGENIC THERAPY

As a tumor grows, there is greater diversity of both the tumor and endothelial cell compartments. Many inhibitors that show potent antiangiogenic activity in prevention of tumor growth work poorly, if at all, at regression of established tumors.[179] This suggests, in part, that an established vasculature differs from newly originating vessels in an incipient tumor. Resistance to antiangiogenic agents can be imagined to occur in several ways. First, agents that are vasculostatic (ie, inhibitors of endothelial cell proliferation and the formation of new blood vessels) could generate a resistant phenotype in tumors by selection. Such agents could include growth factor inhibitors such as antibodies or other drugs against VEGF, bFGF, or angiopoietins. A tumor cell could be envisioned to become independent of this block by relying on other growth factors to stimulate endothelial cell growth.[118] In other words, there is a redundancy in angiogenic growth factors, and as a tumor grows, it may generate one or more of these. Second, agents that induce apoptosis of vascular endothelial cells may potentially lead to resistance by other mechanisms. These particular agents include endostatin, tyrosine kinase inhibitors (SU5416, SU6668), inhibitors of $\alpha_{v\beta3}$ integrin, and others.[68,74,187,225,226] Resistance to these antiangiogenic agents by endothelial cells might develop under epigenetic pressures with changes in expression of surface receptors as a result of tumor microenvironment.[77] Alternatively, upregulation of intracellular survival factors that protect against apoptosis may be an escape route.

Numerous endothelial cell survival factors are being identified. These factors may be able to provide an advantage in growth and survival of endothelial cells exposed to toxic agents. VEGF has been shown to be one of these in a recent report.[89] Tumor cell lines that have acquired resistance to anti–EGF-R antibodies, have been shown to upregulate the expression of VEGF. This increased expression of VEGF also correlated with angiogenic potential in vitro and tumor angiogenesis in vivo. Similarly, the antiapoptotic gene, *survivin*, has been shown to be critical to the regulation of apoptosis in endothelial cells.[227] Targeting this gene using antisense oligonucleotides suppressed the antiapoptotic effect of VEGF on endothelial cells.[228] Manipulating this pathway may be a strategy to diminish endothelial cell viability and potentially enhance the effects of antiangiogenic agents.

The role of tumor suppressor genes or oncogenes may influence how tumors respond to antiangiogenic agents.[229-231] For example, it was recently reported that a tumor suppressor gene, *p53*, can impact survival of tumor cells in conditions of hypoxia. Tumor cells that have inactivation of this gene (seen in a majority of human cancers), have a diminished rate of apoptosis under hypoxic conditions. Such a response can influence the effects of antiangiogenic agents as they starve tumors of a blood supply and generate hypoxic conditions. Therefore, genetic changes in tumor cells may make certain tumors less dependent on angiogenesis.[231]

DISCUSSION

Angiogenesis is an exciting target for novel anticancer therapies because of the many advantages that it may offer. Some of these include accessibility to tumors, independence of tumor cell resistance mechanisms, and broad applicability to many tumor types. Because angiogenesis occurs in very limited circumstances in the adult, biological therapies that target specific receptors, which are upregulated on proliferating endothelium, could potentially be safe and avoid widespread toxicity in the patient.

Further understanding of the biology of angiogenesis and vasculogenesis will improve strategies for targeting endothelial cells for cancer therapy. There are many similarities

between these processes especially growth factors such as VEGF and bFGF, which stimulate endothelial cell proliferation. However, distinct differences exist between blood vessels undergoing vasculogenesis versus sprouting angiogenesis, between blood vessels differentiating into arteries or veins, between blood vessels in different tissue beds, between the vasculature of different tumor types, and between vessels within a single tumor. These differences represent an opportunity to discover new molecules in the regulation of proliferation and differentiation of endothelial cells but also foreshadow possible obstacles when targeting a specific receptor on endothelium for cancer therapy.

Many approaches have been taken to discover novel endothelial surface markers or addresses that define a vascular bed in the liver, versus that of the brain, versus that of another organ. In vivo phage display technologies have shown specific homing peptides for certain organs. These peptides have allowed for novel delivery methods for targeted chemotherapy and radiotherapy. Other strategies have included microarray, serial analysis of gene expression, and proteomic technologies. These may allow for discovery of a whole range of genes and proteins that are over- or underexpressed in endothelial cells undergoing rapid growth in tumors. The patterns of gene expression obtained may provide insight on critical intracellular pathways regulating endothelial cell proliferation. Such genetic approaches are descriptive and will be complemented by functional genomic approaches. Technologies such as retroviral cDNA and peptide library systems will enable investigators to incite distinct perturbations in endothelial cells and then, subsequently, discover novel genes and peptides that could counteract or promote physiologic changes.

There are many factors that have been found to be expressed on both endothelial and tumor cells. Some of these include VEGF and VEGF receptors. Antiangiogenic therapy directed against these targets may result in dual effects on both tumor and endothelial cells. These similarities have led some investigators to speculate that certain tumor cells may have physiologic properties similar to endothelial cells and be able to form primitive vessels.

A wide variety of biological treatments have emerged that target the vasculature. Some of these include gene therapy with antiangiogenesis genes delivered to tumor cells using viral and nonviral methods. Development of newer generation retroviral and lentiviral vectors will enhance delivery of genes to the host for both high level and long-term expression of antiangiogenic factors. The expression of these factors may prove to be critical in chronic suppression of metastatic tumor growth in the host.

Novel imaging and radioimmunotherapeutic strategies are emerging with discovery of new endothelial cell targets. Traditional cancer therapies targeting tumor antigens have often been fraught with the inability to penetrate bulky tumors. Antiangiogenic radioligands may overcome this barrier by acting predominantly in the intravascular compartment. The endothelial cell compartment thus provides the potential advantage of rapid access by therapeutic drugs and imaging agents. With regard to imaging, it may be possible to develop novel PET or single-photon emission computerized tomography methods to diagnose a tumor at an early stage when the angiogenic switch is turned on. These modalities may provide functional insight on the degree of angiogenesis within a tumor. Furthermore, some radioisotopes can be used for both imaging and therapy. As such, antiangiogenic agents coupled to these isotopes may provide a dual mechanism for regressing tumors. In addition to killing endothelial cells (resulting in an indirect bystander effect on the tumor cell compartment) they can also induce direct damage to tumor cells through energy emitted by the radioisotope.

The vasculature can also be targeted by host immune defenses. Proliferating endothelial cell lines can serve as sources of antigens that might mimic the angiogenic endothelium within tumors. Endothelial cell vaccines might be useful in protecting against a variety of tumors akin to a pan-protective cancer vaccine. Similarly, monoclonal antibodies that are specific for angiogenic receptors could be passively administered to prevent further tumor growth at an early stage of disease or for treatment of metastases.

Because of the heterogeneity of the vasculature, it may be advantageous to develop therapies that target multiple critical factors on endothelial cells or, alternatively, to use combination therapies. This may help avoid tumor escape mechanisms or epigenetic resistance mechanisms. Resistance mechanisms to antiangiogenic therapies may develop by the expression of survival factors that would favor continued viability and growth of endothelial cells within a microenvironment. Some of these factors, including VEGF and survivin, will be important targets to reverse potential endothelial cell resistance.

The efficacy of antiangiogenic agents in patients should be assessed quite differently from standard chemotherapies. Here, long-term control of metastases is the goal without the requirement for tumor shrinkage. Determining in vivo biological correlates remains both a challenge and opportunity. Genetic methods will allow for angiogenic profiling of patient tumors and sera for development of customized antiangiogenic therapy. Converting cancer into a chronic disease may take years of assessment. Potential toxicities of these agents to other physiologic or pathophysiologic states of angiogenesis must be considered and carefully monitored. Overall, antiangiogenic

strategies offer a new way of controlling cancer as a chronic disease in combination with existing treatments of surgery, chemotherapy, and radiotherapy.

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Endothelial and smooth muscle cells interact with each other to form new blood vessels. In this review, the cellular and molecular mechanisms underlying the formation of endothelium-lined channels (angiogenesis) and their maturation via recruitment of smooth muscle cells (arteriogenesis) during physiological and pathological conditions are summarized, alongside with possible therapeutic applications.

Mechanisms of angiogenesis and arteriogenesis

PETER CARMELIET

Normal tissue function depends on adequate supply of oxygen through blood vessels. Understanding how blood vessels form has become a principal, yet challenging, objective of the last decade. Unraveling these mechanisms would offer therapeutic options to ameliorate or perhaps even cure disorders that are now leading causes of mortality. In this millenium, we will be required to answer such questions as: Will it be possible to treat ischemic heart disease by stimulating myocardial angiogenesis, and will it be feasible to cure cancer or inflammatory disorders by suppressing excessive vessel growth? Unfortunately, research on angiogenesis has for too long remained descriptive, mainly because the molecular 'players' were not identified. The recent discovery of candidates able to stimulate or inhibit endothelial cells has stirred a growing interest in using these molecules for therapeutic applications. This overview provides an update on the present understanding of the basic molecular mechanisms of how endothelial and smooth muscle cells interact with each other to form blood vessels, as a basis for design of future (anti)-angiogenic treatments.

Development of an endothelium-lined vasculature

Blood vessels in the embryo form through vasculogenesis; that is, through *in situ* differentiation of undifferentiated precursor cells (angioblasts) to endothelial cells that assemble into a vascular labyrinth[1] (Fig. 1). Historically, the term angiogenesis was first used to describe the growth of endothelial sprouts from preexisting postcapillary venules (Fig. 1). More recently, this term has been used to generally denote the growth and remodeling process of the primitive network into a complex network. This involves the enlargement of venules, which sprout or become divided by pillars of periendothelial cells (intussusception) or by transendothelial cell bridges, which then split into individual capillaries (Fig. 1). New vessels in the adult arise mainly through angiogenesis, although vasculogenesis also may occur (Fig. 2). Because vasculogenesis only leads to an immature, poorly functional vasculature, angiogenesis is a therapeutic goal. As the cellular and molecular mechanisms of angiogenesis differ in various tissues (vessels in psoriatic skin enlarge, but they sprout in ischemic retina), the therapeutic stimulation or inhibition of angiogenesis should be adjusted to the target tissue.

Smooth muscle–endothelial cell interactions

Although endothelial cells have attracted most attention, they alone can initiate, but not complete, angiogenesis; periendothelial cells are essential for vascular maturation (Fig. 3). During 'vasular myogenesis', mural cells stabilize nascent vessels by inhibiting endothelial proliferation and migration, and by stimulating production of extracellular matrix (Fig. 1). They thereby provide hemostatic control and protect new endothelium-lined vessels against rupture or regression. Indeed, vessels regress more easily as long as they are not covered by smooth muscle cells[2]; the loss of pericytes around retinal vessels in diabetic patients causes aneurysmal dilatation, bleeding and blindness.

During the subsequent arteriogenesis, vessels become covered by a muscular coat, thereby endowing blood vessels with viscoelastic and vasomotor properties, necessary to accommodate the changing needs in tissue perfusion (Fig. 1). Periendothelial cells also assist endothelial cells in acquiring specialized functions in different vascular beds[3]. Arteriogenesis is recapitulated during the pathological enlargement of preexisting collateral vessels (Fig. 2). Therefore, strategies to promote sustainable and functional new blood vessels should not be restricted to the induction of capillary angiogenesis, but should include the stimulation of arteriogenesis. Likewise, the therapeutic regression of 'muscularized' vessels may require strategies other than the inhibition of endothelium-lined vessels.

Vasculogenesis: the formation of a primitive network

Endothelial and hematopoietic cells share a common progenitor (the hemangioblast). In the yolk sac, hemangioblasts form aggregates in which the inner cells develop into hematopoietic precursors and the outer population into endothelial cells (Fig. 1). Angioblasts may migrate extensively before *in situ* differentiation and plexus formation. Vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR) 2 and basic fibroblast growth factor (bFGF) influence angioblast differentiation[4–7], whereas VEGFR1 suppresses hemangioblast commitment[8]. The molecular mechanisms of how transforming growth factor (TGF)-β1 and TGF-β receptor 2 affect vasculogenesis remain mostly undetermined[9]. Molecules mediating interactions between endothelial cells and matrix macromolecules, fibronectin or matrix receptors (α_5 integrin), also affect vasculogenesis. The $\alpha_v\beta_3$ integrin mediates vasculogenesis in avian but not in murine embryo[10].

Little is known about the mechanisms governing endothelial cell fate: *Ets-1*, *Hex*, *Vezf1*, *Hox* and *GATA* family members, basic helix–loop–helix factors and their inhibitors of differentiation may be involved[11]. Such molecules may be of therapeutic value, as they could determine the 'decision' of endothelial cells to become angiogenic during pathological conditions (called 'angiogenic switch')[12]. The fate of endothelial cells to become integrated into arteries or veins is mediated by the bHLH transcription factor gridlock at the angioblast stage, and, subsequently, by members of the ephrin family, signals that are also involved in guidance of axons and repulsion of neurons[14]. It was once believed that endothelial precursors only exist during embryonic life. However, endothelial precursor cells have been identified in bone marrow and in peripheral blood in adults. VEGF, granulocyte–monocyte colony-stimulating factor, bFGF and insulin-like growth factor (IGF)-1 stimulate their differentiation and mobilization[15,16]. Such precursors colonize angiogenic sites and vascular prostheses in the adult and may hold promise for future therapy (Fig. 2).

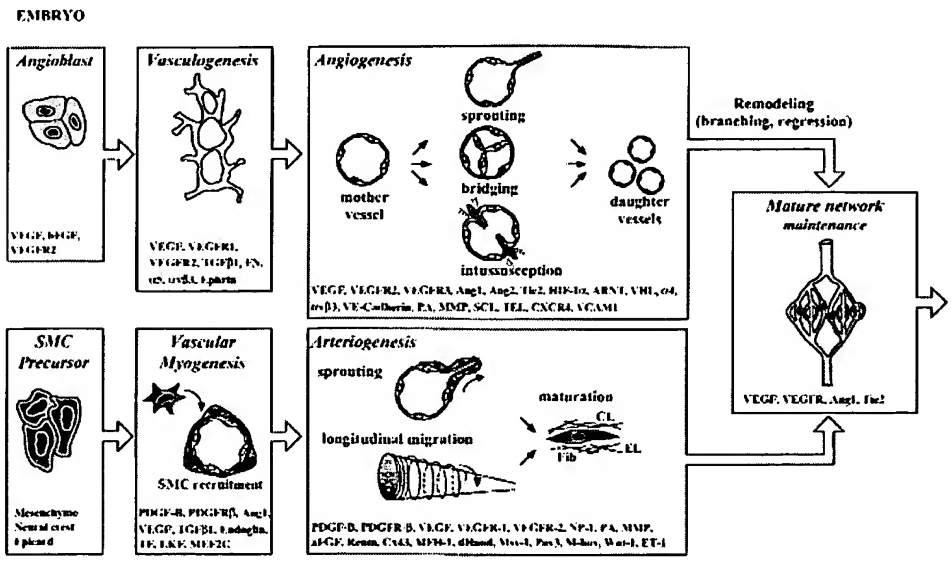

Fig. 1 Endothelial precursors (angioblasts) in the embryo assemble in a primitive network (vasculogenesis), that expands and remodels (angiogenesis). Smooth muscle cells cover endothelial cells during vascular myogenesis, and stabilize vessels during arteriogenesis. CL: collagen; EL: elastin; Fib: fibrillin (Fib).

Angiogenesis: sprouting and remodeling

Angiogenic sprouting is one, but not the only, mechanism of blood vessel formation in the adult; however, it has been studied most extensively. The molecular basis of angiogenesis in the embryo seems to differ from that of pathological angiogenesis in the adult (Figs. 1 and 2). Several steps have been determined (Fig. 3).

Vasodilation, endothelial permeability and periendothelial support: Angiogenesis initiates with vasodilation, a process involving nitric oxide (Fig. 1). Vascular permeability increases in response to VEGF, thereby allowing extravasation of plasma proteins that lay down a provisional scaffold for migrating endothelial cells. This increase in permeability is mediated by the formation of fenestrations, vesiculo–vacuolar organelles and the redistribution of platelet endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial (VE)–cadherin, and involves Src kinases[17]. Although permeability is good for angiogenesis, excessive vascular leakage can be bad and lead to circulatory collapse, intracranial hypertension, formation of adhesion, metastasis, premenstrual discomfort or blindness. Angiopoietin (Ang) 1, a ligand of the endothelial Tie2 receptor, is a natural inhibitor of vascular permeability, tightening preexisting vessels. When acutely administered to adult vessels, Ang1 protects against plasma leakage without profoundly affecting vascular morphology.[18]

For endothelial cells to emigrate from their resident site, they need to loosen interendothelial cell contacts and to relieve periendothelial cell support; that is, mature vessels need to become destabilized (Fig. 3). Ang2, an inhibitor of Tie2 signaling, may be involved in detaching smooth muscle cells and loosening the matrix[14,19]. Proteinases of the plasminogen activator, matrix metalloproteinase (MMP), chymase or heparanase families influence angiogenesis by degrading matrix molecules and by activating or liberating growth factors (bFGF, VEGF and IGF-1), sequestered within the extracellular matrix[20]. Urokinase-type plasminogen activator (u-PA) is essential for revascularization of myocardial infarcts[21], whereas antagonists of the u-PA receptor inhibit tumor angiogenesis. MMP-3, MMP-7 and MMP-9 affect angiogenesis in neonatal bones[22] and tumors[23], whereas tissue inhibitors of MMPs 1 or 3) or PEX, the naturally occurring fragment of MMP-2, by preventing binding of MMP-2 to $\alpha_v\beta_3$, inhibit tumor angiogenesis[24]. Paradoxically, tissue inhibitor of MMPs 1 and plasminogen activator-inhibitor 1 are risk factors for a poor prognosis in cancer patients and promote tumor angiogenesis in mice by preventing excessive proteolysis (Bajou *et al*, manuscript submitted)[23]. In support of this, endostatin inhibits tumor angiogenesis by increasing plasmin generation (A. Reijerkerk *et al.*, manuscript submitted).

Endothelial cell proliferation and migration: Once the path has been cleared, proliferating endothelial cells migrate to distant sites. VEGF (ref. 4), placental growth factor (PLGF), VEGF-B, VEGF-C, VEGF-D and their receptors VEGFR2, VEGFR3 (ref. 25) and neuropilin-1 (a co-receptor of VEGFR2; ref. 26) have specific functions: VEGF and its receptor VEGFR2 affect embryonic, neonatal and pathological angiogenesis and are therapeutic targets, although much remains to be learned about the involvement of the distinct VEGF isoforms or of the heterodimers of VEGF family members[4–6,27]. $VEGF_{120}$ alone initiates but does not complete angiogenesis[28]. VEGFR3 is involved in embryonic angiogenesis[29] and is expressed in pathological angiogenesis, whereas VEGF-C (a ligand of VEGFR3) is angiogenic in adult pathology[30]. The angiogenic or lymphangiogenic activity of VEGF-C depends on its processing. Truncation of VEGFR1 at the tyrosine kinase domain does not impair embryonic angiogenesis, but the involvement of VEGFR-1 signaling during pathological angiogenesis remains undetermined[31]. Indeed, the loss of PLGF specifically impairs pathological but not physiological angiogenesis, by increasing the responsiveness of VEGFR2 to VEGF through increased VEGFR2 tyrosine phophorylation (P.C., manuscript submitted). Loss of VEGF-B affects coronary function after coronary occlusion. Ang1 phosphorylates tyrosine in Tie2 and is chemotactic for endothelial cells, induces sprouting and potentiates VEGF, but fails to induce endothelial proliferation[14,32]. In contrast to VEGF, Ang1 itself does not initiate endothelial network organization, but stabilizes networks initiated by VEGF, presumably by stimulating the interaction between endothelial and periendothelial cells. This indicates that Ang1 may act at later stages than VEGF (Fig. 3)[14,32]. Ang2, at least in the presence of VEGF, is also angiogenic. However, more recent data indicate that overexpression of Ang1 in tumors suppresses their growth, but whether this means that Ang1 acts physiologically to promote angiogenesis by inducing vessel maturation and stabilization, but that this function may inhibit growth of immature and stable tumor vessels, remains to be determined (P. Maisonpierre, personal communication). Low

levels of phosphorylated Tie2 have been detected in the adult quiescent vasculature, indicating involvement of Tie2 in vascular maintenance.

Members of the fibroblast growth factor and platelet-derived growth factor (PDGF) family are redundant during normal development[33,34], but they affect angiogenesis when administered, probably by recruiting mesenchymal or inflammatory cells. TGF-β1 and tumor necrosis factor (TNF)-α can either stimulate or inhibit endothelial growth, and may be involved in tumor dormancy[35]. Molecules involved in cell–cell or cell–matrix interactions, such as the $\alpha_v\beta_3$ integrin, which localizes MMP-2 at the endothelial cell surface, mediate endothelial spreading, explaining why $\alpha_v\beta_3$ antagonists inhibit angiogenesis[36]. PECAM-1 and EphrinB2 (G. Yancopoulos, personal communication) may also be involved in pathological angiogenesis. Nitric oxide, a downstream effector of VEGF, TGFβ-1 and other angiogenic factors, is not essential for embryonic vascular development, but affects pathological angiogenesis and improves the reendothelialization of denuded vessels[37]. A growing list of molecules is being discovered that are angiogenic after exogenous administration, but whose endogenous angiogenic function remains undetermined: erythropoietin, IGF-1, neuropeptide-Y, leptin, Thy-1, epidermal growth factor, tissue factor (initiator of blood coagulation), hepatocyte growth factor, interleukins hormones and chemokines.

Angiogenic sprouting is controlled by a balance of activators and inhibitors. Angiogenesis inhibitors suppressing the proliferation or migration of endothelial cells include angiostatin (an internal fragment of plasminogen)[38], endostatin (a fragment of collagen XVIII; ref 39), antithrombin III, interferon-β, leukemia inhibitory factor and platelet factor 4. Naturally occurring angiogenesis inhibitors may be involved in tumor dormancy and are being tested for anti-cancer treatment.

Lumen formation: Endothelial cells often assemble as solid cords that subsequently acquire a lumen. Intercalation or thinning of endothelial cells and fusion of preexisting vessels allow vessels to increase their diameter and length. In contrast to normal vessels, tumor vessels are often abnormally enlarged, but blood flow in tumor vessels is often chaotic, slow and not efficient in meeting metabolic demands[40]. $VEGF_{189}$ decreases luminal diameter, whereas $VEGF_{121}$, $VEGF_{165}$ and their receptors increase lumen formation, in addition to increasing vessel length. In certain tissues (such as psoriatic skin), VEGF mainly exerts a morphogenetic activity by enlarging existing vessels. Ang1 in combination with VEGF also increases luminal diameter[32]. Other molecules affecting lumen formation are integrins ($\alpha_v\beta_3$ or α_5) and the myocyte enhancer binding factor 2C (MEF2C) transcription factor. Excessive proteolysis may lead to cystic assembly of endothelial cells and prevent tube formation. Thrombospondin (TSP)-1 is an endogenous inhibitor of lumen formation.

Endothelial survival: Once assembled in new vessels, endothelial cells become quiescent and survive for years. The importance of endothelial survival is demonstrated by findings that reduced survival causes vascular regression in the embryo[41]. Endothelial apoptosis is a natural mechanism of vessel regression in the retina and ovary after birth and a frequent effect of (therapeutic) inhibitors of angiogenesis. Endothelial apoptosis is induced through deprivation of nutrients or survival signals when the lumen is obstructed by spasms, thrombi or the shedding of dead endothelial cells, or when a change in the angiogenic gene profile occurs[27,28,42]. For example, exposure of premature babies to hyperoxia reduces VEGF levels and causes vessel regression in the retina[43]. The survival function of VEGF depends on an interaction between VEGFR2, β-catenin and vascular endothelial (VE)–cadherin[41]. Ang1 also promotes, whereas Ang2 suppresses, endothelial survival, at least in the absence of angiogenic stimuli (Fig. 3), and has been suggested to contribute to the regression of 'co-opted' tumor vessels[14,32,44] or of hyaloid vessels (G. Yancopoulos, personal communication). Disruption of the interaction with matrix macromolecules, using $\alpha_v\beta_3$ antagonists or the desintegrin accutin, also results in endothelial apoptosis, but, as $\alpha_v\beta_3$ is only expressed in proliferative cells, pre-existing quiescent blood vessels remain unaffected[36]. Different vascular beds may have specific survival mechanisms, such as brain-derived neurotrophic factor for coronary endothelial cells (B. Hempstead, personal communication). Hemodynamic forces are essential for vascular maintenance, as physiological shear stress reduces endothelial turnover and abrogates TNF-α mediated endothelial apoptosis. Endothelial apoptosis can be also induced by nitric oxide, reactive oxygen species, angiostatin, TSP-1, the metallospondin METH-1, interferon-γ, tissue factor pathway inhibitor and vascular endothelial growth inhibitor (VEGI).

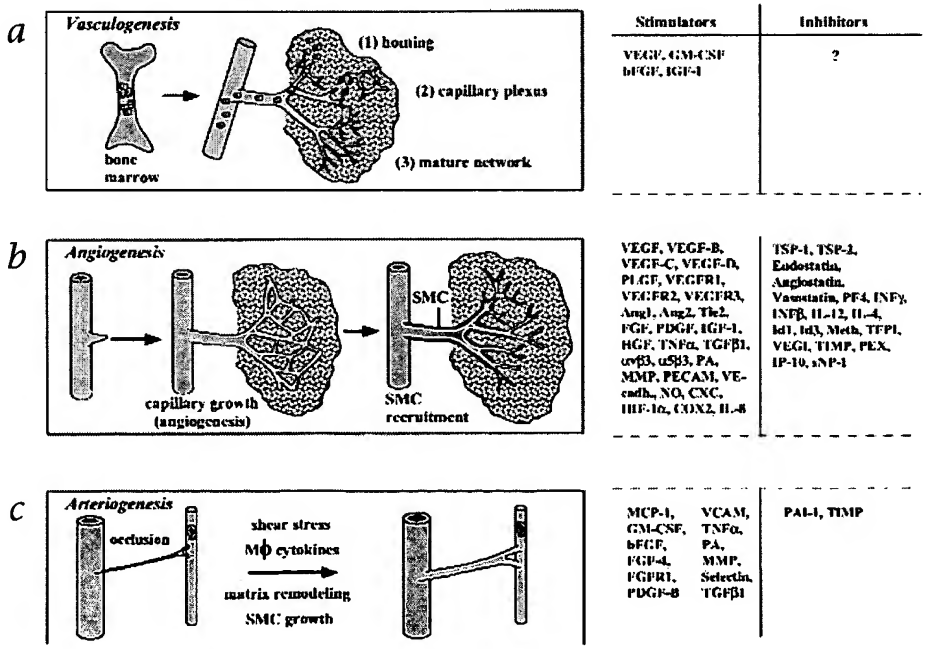

Fig. 2 Pathological vascular growth in the adult may occur via vasculogenesis (angioblast mobilization), angiogenesis (sprouting) or arteriogenesis (collateral growth).

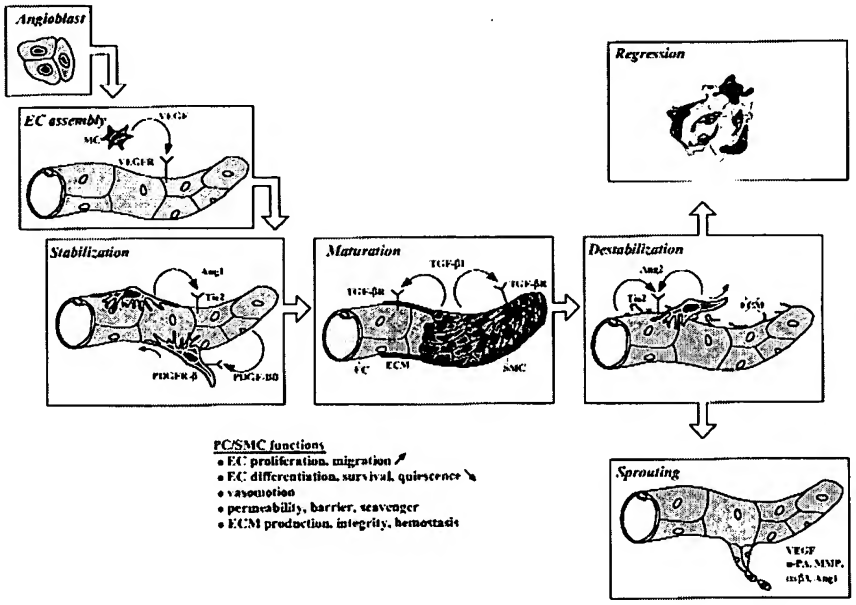

Fig. 3 VEGF initiates assembly of endothelial cells (EC), PDGF-BB recruits pericytes (PC) and smooth muscle cells (SMC), whereas angiopoietin-1 (Ang1) and TGF-b1 stabilize the nascent vessel. Angiopoietin-2 (Ang2) destabilizes the vessel, resulting in angiogenesis in the presence of angiogenic stimuli, or in vessel regression in the absence of endothelial survival factors.

Several endothelial survival factors (VEGF, Ang1 and $\alpha_v\beta_3$) suppress p53, p21, p16, p27 and *Bax*, whereas they variably activate the survival PI3-kinase/Akt, p42/44 mitogen-activated protein kinase, Bcl-2, A1 and survivin pathways. The mechanism of action remains unknown for many other angiogenesis inhibitors, including prothrombin kringle-1 and kringle-2, TSP-2, PECAM-1 antagonists, interleukins 4 and 12, interferon-α, cyclooxygenase-2 (Cox2)-inhibitors, 1,25-dihydroxyvitamin-D_3 and the N-terminal fragment of prolactin. The transcription factor *Braf* may be involved in endothelial survival.

Endothelial differentiation: To accommodate local physiological requirements, endothelial cells acquire specialized characteristics that are determined in part by the host tissue[45]. For example, an interaction of astroglial cells expressing glial fibrillary acidic protein, pericytes and normal angiotensinogen levels is essential for development of the blood–brain barrier[34]. In contrast, endothelial cells in endocrine glands, involved in the exchange of particles, become discontinuous and fenestrated; this is possibly mediated by interactions between VEGF and the extracellular matrix. Endothelial cells in tumors are abnormal in many ways: They are multilayered, protrude extensions bridging and splitting vessels, contain intercellular and transcellular holes, show relatively uncontrolled permeability and undergo constant remodeling. The recent and controversial finding that tumor vessels are 'mosaic' and are lined by both endothelial cells and malignant 'vasculogenic' tumor cells (or 'vasculogenic mimicry') may have considerable consequences for anti-angiogenesis tumor therapy (ref. 46 and R. Jain, personal communication). Epitopes specific for tumor endothelial cells are attractive targets for the 'homing' of pro-apoptotic or thrombotic molecules in anti-cancer therapy[47]. Microenvironmental factors also determine the endothelial barrier in tumors, as endothelial cells contain more fenestrae when tumors are grown under the skin than in the brain.

Remodeling: So far, very little is known about the spatial cues guiding endothelial cells into correct patterns and three-dimensional networks, a goal for therapeutic angiogenesis. Maturation of the endothelial network involves remodeling and 'pruning' capillary-like vessels with uniform size, and irregular organization into a structured network of branching vessels (Fig. 1). Intussusception, resulting in replacement of vessels by matrix, underlies 'pruning' and branching. Gene inactivation studies indicate a morphogenetic function for the distinct VEGF isoforms and VEGFR3 (refs. 5, 6, 28, 29), the endothelial 'orphan' receptor Tie1 (ref. 48), the T-cell-leukemia protein stem cell leukemia factor/tal-1, *TEL* (a member of the Ets family of transcription factors), the GTP-binding protein $G\alpha_{13}$, Jagged, chemokine receptor 4, vascular cell adhesion molecule 1, α_4 integrin and fibronectin. Recent studies in zebrafish have identified the candidate gene responsible for *gridlock*, a vascular patterning defect resembling coarctation of the aorta[13]

Vascular myogenesis

Smooth muscle cell fate: Smooth muscle cells have a complex origin depending on their location (Fig. 1). A puzzling question, therefore, is whether distinct growth factors/receptors mediate their fate in different vascular beds. This would have substantial therapeutic consequences, as stimulating collateral vessels in the heart or limb would then require the use of different therapeutic factors. This idea is supported by recent findings that *Bves* is a specific marker for coronary vascular smooth muscle cells, whereas brain-derived neurotrophic factor is a specific survival factor for coronary endothelial cells. Smooth muscle cells can transdifferentiate from endothelial cells, differentiate from mesenchymal cells *in situ* in response to as-yet-unidentified endothelial-derived stimuli, or from bone marrow precursors or macrophages. Smooth muscle cells of the coronary veins are derived from atrial myocardium, whereas mural cells of the coronary arteries are recruited from the epicardial layer[49]. The large thoracic blood vessels, which are often affected by congenital malformations, contain smooth muscle cells, derived from cardiac neural crest cells[50]. Smooth muscle fate involves transcriptional control by the serum response factor, *Prx-1* and *Prx-2*, *CRP2/SmLIM*, capsulin, and members of the *Hox*, *MEF2* and *GATA* family.

Smooth muscle cell recruitment and growth: PDGF-BB is a chemoattractant for smooth muscle cells[34] (Fig. 1). VEGF also promotes mural cell accumulation, presumably through the release of PDGF-BB or binding to VEGF receptors[2,28]. Ang1 and Tie2 affect growth and maintenance of blood vessels by stabilizing the interaction of mural cells with nascent endothelial channels, and by inducing branching and remodeling[14,19,33,51]

(Fig. 3). Hereditary dysfunction of Tie2 in humans induces vascular malformations, characterized by vessels with fewer smooth muscle cells. TGF-β1, TGF-βR2, endoglin (an endothelial TGF-β binding protein) and Smad5 (a downstream TGF-β signal) are involved in vessel maturation in a pleiotropic manner: they inhibit endothelial proliferation and migration, induce smooth muscle differentiation and stimulate extracellular matrix production, thereby 'solidifying' the endothelial–mural cell interactions[9,52] (Fig. 3). Patients lacking endoglin suffer hereditary hemorrhagic telangiectasia type 1. N-cadherin seems to 'glue' endothelial and mural cells in close apposition. Endothelin-1, produced by endothelial cells of thoracic blood vessels, is chemotactic for neural crest cells, transforming into smooth muscle cells[53]. Tissue factor of coagulation promotes pericyte recruitment, possibly through the generation of thrombin and/or a fibrin-rich scaffold. Other candidates are heparin-binding epidermal-growth-factor-like factor and the transcription factors *LKLF*, *COUP-TFII* and *MEF2C* (ref. 54). Ang2 and EphrinB2 are also expressed by vascular smooth muscle cells in particular tissues, but their involvement remains undefined (G. Yancopoulos, personal communication).

Arteriogenesis

Smooth muscle cell migration and growth: Once mural cells have been recruited, they further 'muscularize' the nascent vasculature by sprouting or by migrating alongside preexisting vessels, using these as guiding cues (Fig. 1, longitudinal migration), such as in the retina or in the heart where smooth muscle coverage proceeds in a epicardial-to-endocardial direction. In mesenchyme-rich tissues, such as in the lung, *in situ* differentiation of mesenchymal cells contributes to muscularization. Presumably, signals similar to those mediating smooth muscle cell recruitment and growth during initial vascular myogenesis are involved in arteriogenesis. Fibroblast growth factors may be involved in branching of coronary arteries, whereas the renin-angiotensin system may be involved in initiation, branching, and elongation of the renal arterial tree[55].

A pathological type of arteriogenesis is the 20-fold enlargement of preexisting collateral arterioles after occlusion of a supply artery[56] (Fig. 2). As a result of the increased collateral flow, endothelial cells express monokines (monocyte chemotactic protein 1) and monocyte adhesion molecules (such as intracellular adhesion molecule 1). The recruited monocytes infiltrate the vessel wall and destroy the media, using proteinases and death factors (TNF-α) (ref. 56). Activated endothelial cells then upregulate bFGF, PDGF-B and TGF-β1, thereby inducing the regrowth of smooth muscle cells and vessel enlargement. Arteriogenesis is impaired in *op/op* mice lacking macrophage colony-stimulating factor. A deficiency in PLGF prevents collateral growth by impairing monocyte recruitment, extravasation of fibronectin and smooth muscle cell growth (P.C., manuscript submitted).

Smooth muscle cell differentiation: Mural cells acquire specialized characteristics, including contractile components (Fig. 1). Loss of the intermediate filament desmin results in smooth muscle hypoplasia and degeneration, whereas a deficiency in MEF2C results in impaired smooth muscle differentiation[54]. Interstitial matrix components provide the developing arteries viscoelastic properties (elastin and fibrillin-2) and structural strength (collagen and fibrillin-1). A deficiency in these components, as in gene-inactivated mice or in humans with hereditary Marfan syndrome or atherosclerotic media destruction, results in weakening and aneurysmal dilatation of the arteries[57]. However, elastin also (negatively) regulates smooth muscle cell growth, as mice or humans lacking elastin die of obstructive intimal hyperplasia[58]. During pathological conditions (such as restenosis or atherosclerosis), smooth muscle cells often 'de-differentiate' to an embryonic phenotype, reverting from their 'contractile' to a 'synthetic' phenotype.

Remodeling: The large thoracic vessels undergo considerable remodeling during development. Genetic analysis has shown that loss of MFH-1, dHand or Msx1, Pax-3, Prx1, retinoid acid receptors, the neurofibromatosis type-1 gene product, Wnt-1, connexin 43 or endothelin-1 (ref. 53) induce aortic arch malformations. Prostaglandins mediate closure of the neonatal ductus arteriosus[59]. Signals involved in neuronal patterning also seem to be involved in vascular patterning. In the avian heart, there is a close spatial juxtaposition between coronary arteries and Purkinje cells of the myocardial conduction system. Endothelin-1, locally generated in the coronary artery, is an instructive cue for the differentiation of cardiomyocytes into Purkinje cells[60]. Loss of semaphorin-3C (J. Epstein, personal communciation) or of neuropilin-1, a receptor for neurorepulsive semaphorins, induces abnormal patterning of the large thoracic vessels[61]. Arterial rarefication also occurs during pulmonary or systemic hypertension. An imbalance between endothelin-1 and nitric oxide initially induces vasospasms, but when sustained, this progresses to irreversible vascular loss. Loss of PLGF or u-PA protects against pulmonary vascular remodeling (P.C., manuscript submitted).

Modulation of vascular growth

Involvement of hypoxia and nutrients: Hypoxia-inducible transcription factors (HIF-1β, HIF-1α and HIF-2α) trigger a coordinated response of angiogenesis and arteriogenesis by inducing expression of VEGF, VEGFR1, VEGFR2, neuropilin-1, Ang2, nitric oxide synthase, TGFβ-1, PDGF-BB, endothelin-1, interleukin-8, IGF-II, Tie1, cyclooxygenase-2 and so on[62]. The von Hippel Lindau tumor suppressor gene product suppresses expression of hypoxia-inducible target genes during normoxia. Gene inactivation studies have shown that angiogenesis, not vasculogenesis, is regulated by hypoxia[62,63]. Tumors lacking HIF-1β or HIF-1α fail to develop vascularization and lack hypoxic induction of VEGF expression[64], whereas stabilization of HIF-1α by peptide regulator 39 induces angiogenesis in the myocardium[65]. Hypoxia-inducible factors and hypoxia-response elements are now being tested for angiogenic (gene) therapy of tissue ischemia. Metabolic stimuli, including hypoglycemia and low pH, also stimulate vessel growth, but their mechanisms remain to be determined.

Involvement of mechanical factors: Vasculogenesis occurs mostly independently, whereas angiogenesis coincides with the onset of and is influenced considerably by flow. As a result of the higher blood pressure in the capillaries proximal to the aorta, coronary arteries become covered by smooth muscle cells at earlier times than do veins[66]. Remodeling of the developing thoracic arteries or of collateral vessels after arterial occlusion also depends on flow[56]. Gene inactivation studies have shown that shear-stress-induced vascular remodeling is affected by nitric oxide and P-selectin, that the response of resistance arteries to flow is determined by vimentin, and that vascular tone is affected by bFGF (ref. 33). Mechanical forces modulate vascular function through shear-stress-responsive gene transcription.

Lymphangiogenesis

The molecular mechanisms governing lymphangiogenesis remain mostly undefined because of the lack of specific molecular markers. Lymphatic vessels sprout from VEGFR3-expressing veins at a time when fluid extravasated by the increased blood pressure needs to be reabsorbed. VEGF-C is lymphangiogenic, as demonstrated by the lymphatic hyperplasia and sprouting that occurs after administration or transgenic overexpression of this molecule[67]. A similar although less-potent function has been recently identified for VEGF-D (K. Alitalo, personal communication). LYVE-1 (lymphatic vessel endothelial hyaluronan receptor) and VEGFR3 mark lymphatic vessels in the embryo and the adult[25], and genetic mutations of VEGFR3 have been identified in patients with herediatry lymphoedema (K. Alitalo, personal communication). *Prox-1* transcriptionally regulates lymphatic sprouting and budding[68]. Gene targeting studies also indicate involvement of Ang2 in lymphatic development (G. Yancopoulos, personal communication).

Physiologic or pathologic angiogenesis: different or alike?

Growth of new blood vessel in the adult occurs through vasculogenesis (mobilization of bone marrow-derived endothelial stem cells), angiogenesis or arteriogenesis[56] (Fig. 2). Several mechanisms mediating pathological blood vessel growth resemble those during embryogenesis. However, evidence is emerging that distinct mechanisms may govern adult blood vessel formation, although some of these apparent differences may reflect our incomplete understanding (Figs. 1 and 2). The requirement for different signals is not unexpected, given that endothelial cells are loosely connected and actively growing in the embryo, whereas they are quiescent and encapsulated by a thick mural coat in the adult. Examples of molecules not or minimally involved in embryonic vascular development but substantially affecting pathological angiogenesis include Cox2, PLGF (P.C., manuscript submitted), $\alpha_v\beta_3$ (ref. 36), proteinases[21], plasminogen activator inhibitor 1 (ref. 23), nitric oxide[37] and TSP-2. Many stimulators and inhibitors affect adult blood vessel formation, although we do not understand their functions before birth, if indeed they have any at all. Another difference between physiological or pathological angiogenesis, is that the latter is often induced by (some degree of) inflammation. Monocytes/macrophages, platelets, mast cells and other leukocytes are 'chemoattracted' to sites of inflammation or wound healing, in part by angiogenic factors such as VEGF. These blood-borne cells produce angiogenic and arteriogenic factors (VEGF, bFGF, TGFβ-1, interleukin-8, PDGF, IGF-1, monocyte chemotactic protein 1, TNF-α and proteinases) that, in turn, attract endothelial and smooth muscle cells, fibroblasts, leukocytes or platelets[20,56,69].

Therapeutic consequences, questions and perspectives

The recent insights in the molecular basis of angiogenesis have resulted in treatment paradigms to promote or inhibit angiogenesis. Although these approaches are in their infancy, they are promising. However, because of the rapid evolution and enthusiasm of the field, angiogenic molecules are often being tested in clinical trials, without a complete understanding of their mechanism of action. In addition, many questions remain unanswered. For example, is administration of a single angiogenic molecule sufficient? $VEGF_{120}$ alone initiates but does not complete angiogenesis and arteriogenesis in transgenic mice[28], and mice expressing $VEGF_{164}$ alone are more normal than $VEGF_{120}$ mice; transgenic overexpression of VEGF and Ang1 induces more-numerous and more-stable, yet more-irregular, vessels[14,32]. If one is not sufficient, how should angiogenic molecules be administered in combination: simultaneously or sequentially? Will it be feasible to administer a potent molecule like VEGF in quantitities sufficient for therapy without causing toxicity (hypotension, edema), and, conversely, how do we explain the beneficial effect of administration of minimal VEGF? Are the other VEGF homologs (PLGF, VEGF-B or VEGF-C) attractive, perhaps safer, angiogenic targets? Are Ang1 and Ang2 molecules that stimulate, stabilize or suppress vessels, or does their pro- or anti-angiogenic activity depend on the microenvironment, tissue and context? Are hypoxia-inducible factors safe 'master switches' to use for therapeutic angiogenesis, given their possible involvement in cell death[64]? How much do bone marrow-derived endothelial precursors contribute to pathological angiogenesis, or is local proliferation of angiogenic endothelial cells *in situ* more important? Which (anti)-angiogenic gene therapy methods and routes should be used to avoid infiltration of pro-angiogenic inflammatory cells? Do we deliver angiogenic drugs to the non-ischemic or peri-ischemic myocardium? How long should angiogenic factors be administered: will therapeutic angiogenesis lead to a sustainable and functional vasculature or will vessels regress upon ending therapy? Should treatment not be targeted to arteriogenesis instead of angiogenesis, and will therapeutic angiogenesis/arteriogenesis only succeed in ischemic regions? Will we be able to extrapolate to patients the success of angiogenesis inhibitors (endostatin, angiostatin, vasostatin, thrombospondin, troponin, MMP-inhibitors and so on) in murine models of (ectopic) tumor implantation? Are proteinase inhibitors a good choice to suppress angiogenesis, given the poor prognostic value of plasminogen activator inhibitor 1 in cancer patients[23], or instead should we use molecules (like endostatin) that increase plasmin proteolysis? How much do we need to understand of the mechanism of action of (anti)-angiogenic candidates and/or become aware of their side effects before initiating clinical trials: will therapeutic angiogenesis promote growth of dormant tumors or atherosclerotic plaques; will inhibition of tumor angiogenesis be feasible when tumor vessels are mosaically lined by tumor cells and tumors easily find escape routes to switch on alternative angiogenic programs? Considering the link between angiogenesis and neurogenesis[14], and the recent finding that VEGF improves ischemic neuropathy[70], will long term (anti)-angiogenic treatment cause undesired neuronal effects? Finally, should angiogenic treatment be tailored to individual patients and require genetic prescreening? Indeed, recent genetic studies in mice indicate that loss of u-PA prevents therapeutic myocardial angiogenesis with VEGF (ref. 21). Furthermore, angiogenesis differs more than 10-fold in mice of various genetic backgrounds, and some strains respond by angiogenic sprouting, wereas others by morphogenetic remodeling[71]. Clearly, more work is needed in the future, but—at least—the outlook has become a promising one.

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Independent Association of Angiogenesis Index with Outcome in Prostate Cancer

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ABSTRACT

New molecular factors have been characterized that are associated with the prognosis of prostate carcinoma patients, including p53 status and angiogenesis. We reported recently that mutant p53 (mp53) was associated with decreased expression of an endogenous inhibitor of angiogenesis, thrombospondin-1 (TSP-1), and increased microvessel density in melanoma and breast cancer. In this study, we performed a similar analysis on primary prostate carcinoma to determine whether these factors were associated with each other or patient outcomes. Paraffin-embedded specimens of 98 cases of primary prostate carcinoma were obtained and examined to confirm tissue diagnosis and Gleason scores. Carcinoma-specific levels of p53, TSP-1, and tumor angiogenesis were determined using semiquantitative immunohistochemistry (IHC) methods. Acquisition of mp53 was significantly associated with decreased TSP-1 ($P = 0.002$) and increased angiogenesis ($P < 0.0001$). An angiogenesis index integrating mp53, TSP-1, and angiogenesis (CD31) scores was found to be an independent predictor of survival in univariate and multivariate analyses that included Gleason score, clinical stage, and patient age. Further validation of the angiogenesis index in prostate carcinoma may provide a new tool to stratify patient risk.

INTRODUCTION

A wide variety of clinical and pathological features are currently used to assess risk and tailor treatment strategies for patients with nonmetastatic prostate cancer, including patient age, comorbid conditions, primary tumor stage, prostate-specific antigen level, and Gleason score (1, 2). Clinical tumor stage and Gleason scores have been important predictive factors for local tumor control, as well as disease-free and overall survival. However, molecular prognostic markers that could further stratify prostate carcinoma patients into high-, intermediate-, or low-risk categories for dissemination and survival are lacking. Integration of molecular profiles with conventional pathological staging techniques may provide prognostic information enabling the clinician to accurately predict a given patient's risk of dissemination. Such information could refine the range of treatment options most suited to an individual patient's recurrence risk.

Mutation of the *p53* tumor suppressor gene has been implicated in the tumorigenesis of various neoplasms, including prostate cancer (3–6). Clinically relevant functions of wt[2] p53 include cell cycle regulation and modulation of apoptosis. Recent studies suggest that p53 may also be involved in upregulating the expression of TSP-1, a cell matrix adhesion glycoprotein that acts as an inhibitor of tumor angiogenesis and metastasis. Qian *et al.* (7) showed an inverse correlation between expression of TSP-1 in the stroma of pancreatic adenocarcinoma and that of mp53. Grossfeld *et al.* (8) demonstrated that bladder carcinoma outcomes were related to mp53, TSP-1, and angiogenesis, and that the three factors were interrelated. We have found a similar correlation between acquisition of mp53, loss of TSP-1, and increased angiogenesis in melanoma and breast cancer specimens (9, 10). In this study, we evaluated 98 cases of primary prostate carcinoma for expression of mp53, TSP-1, and angiogenesis to determine whether there was a significant association between these markers and stage, Gleason score, or clinical outcome. We report here that an AI integrating mp53, TSP-1, and angiogenesis scores was strongly associated with survival in both univariate and multivariate analysis that included stage, Gleason score, treatment status, and patient age. The AI was also associated with the degree of tumor differentiation as measured by Gleason scores.

MATERIALS AND METHODS

Patient Material. Paraffin blocks of prostatectomy (radical or transurethral) or needle biopsy specimens obtained from the pathology departments of the University of California, San Francisco and Western Medical Center were evaluated. Serial blocks for 98 cases that included a broad clinical spectrum of patients were evaluated (Table 3). The median patient age was 70 years. Survival data from the time of diagnosis were obtained from the tumor registry or patient chart review. Median follow-up was 7.5 years. A pathologist blinded to clinical charac-

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[2] The abbreviations used are: wt, wild type; TSP-1, thrombospondin-1; mp53, mutant p53; IHC, immunohistochemistry; AI, angiogenesis index; HScore, histoscore.

teristics and clinical outcome examined all tissue sections to confirm the diagnosis and to determine the Gleason score. The 1992 American Joint Committee on Cancer staging system was applied to these cases for outcomes analysis.

IHC. Immunohistochemical detection of p53, TSP-1, and microvessel counts was performed (7). Five-μm tissue sections of fixed, paraffin-embedded specimens were cut, mounted on poly-L-lysine slides (VWR Superfrost Plus), and then deparaffinized in Histoclear. Specimens were rehydrated by sequential washing in ethanol solutions. Antigen retrieval for CD31 used Pronase digestion for 20 min or microwave boiling in citrate buffer for 15 min for p53. No antigen retrieval was necessary for TSP-1 detection. Slides were then incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity and rinsed in tap water, followed by distilled water. Each slide was subsequently incubated in 100 μl of goat serum (Protein Block; Biogenex, San Ramon, CA) for 10 min at room temperature. Excess blocking buffer was shaken off; the slide was incubated with primary antibody solution for 30 min at room temperature and then rinsed twice in PBS for 5 min. Tissue sections were then incubated in biotinylated goat anti-mouse immunoglobulin for 20 min at room temperature in a humidified chamber. After rinsing for 5 min in PBS, each section was exposed to peroxidase-conjugated streptavidin and incubated for 20 min at ambient room temperature, rinsed in PBS, and exposed to diaminobenzidine for 3 min. Slides were then rinsed in PBS for 5 min, exposed to hematoxylin for 1 min, rinsed for 10 min in tap water, dehydrated in ascending ethanol series, cleared in xylene, coverslipped in Permount, and viewed at ×40. All IHC procedures were optimized in preliminary experiments. All antibody reagents were commercially available; p53 was detected with antibody DO1 (Santa Cruz Biotechnology, Santa Cruz, CA); TSP-1 was detected with clone p12 (Immunotech, Marseille, France), and CD31 was detected with clone JC/70A (Dako, Carpinteria, CA). Paraffin-embedded MCF-7 40F and MCF-7 wt breast cancer cells were used as positive controls for p53 and TSP-1 IHC staining, respectively. Titrations were performed for all antibody reagents to insure minimal background staining and optimal antigen detection. Invasive breast carcinoma specimens that stained negative for p53 or TSP-1 were used as negative controls. Control cell line expression status was confirmed previously by Western blot techniques (9). Positive p53 immunostaining has been reported to be related to p53 mutations that increase p53 protein half-life, leading to intracellular accumulation (5). We therefore refer to immunodetectable p53 as mp53 in this report. However, the sensitivity and specificity of IHC for detection of mutant forms of p53 is ~80%, indicating that not all mutant forms can be detected and that not all cases of detectable overexpression of p53 protein are related to mutations. Entire tissue sections were evaluated for intensity and percentage of tumor cells staining positively. Only the malignant component was scored for each case. The intensity of p53 staining was scored as follows: negative when <5% of tumor cells displayed staining; 1+ when intensity was mild; 2+, moderate; 3+, when intensity was equal to the positive control; and 4+ when intensity was greater than the positive control (9, 11). The 5% cutoff used to delineate a positive staining result was based on the previously published cutoff values by Grant *et al.* (9) in melanoma, and Poller *et al.* (11) in mammary ductal carcinoma *in situ* and on observations that tumors showing only weak focal p53 protein expression had expression of p53 equivalent to that sometimes seen in normal cutaneous basal cells. HScores were assigned on the basis of multiplying the percentage of cells staining positive by the intensity of staining plus 1 [HScore = % positive × (intensity + 1)]. HScores are an objective measurement of tumor heterogeneity reflecting the variability in percentage and intensity of staining within a tumor and provide a single unit of measure for the amount of marker present in the field examined, as originally described in early work to quantitate estrogen receptor content in breast cancer by IHC (12). All procedures were performed by pathologists who had no previous knowledge of the clinical outcomes for this series of cases (A. K., H. K., and R. S.). Statistical analysis was performed independently (C. M., K-T. L., T. K.).

Angiogenesis controls with intermediate vessel counts were run in parallel with each series of slides to insure appropriate CD31 staining. An invasive breast carcinoma specimen with high microvascular staining was used for the positive control, and the nonstaining areas in the same tumor tissue were used for the negative control. Light microscopy was used to identify three regions within or immediately adjacent to the tumor that contained the greatest microvessel density ("hotspots"). Microvessel counts were then performed using a ×200 field within the designated hotspot (9, 13, 14). Of the three areas where the highest number of discreet microvessels were stained, the area of greatest counts was chosen for scoring. Any immunoreactive endothelial cell that was separate from adjacent microvessels was considered a "countable" vessel.

Image Analysis. On the basis of the intracellular and extracellular localizations of TSP-1, tumor expression levels of TSP-1 were measured by image analysis as described previously to integrate overall tissue expression levels (9). Briefly, we used a CAS 2000 two-color system (Becton Dickinson, San Jose, CA) that used a light microscope attached to an interactive microcomputer capable of high-speed digital image processing for cell measurements. Image channels were matched to two-component immunohistochemical staining to enhance the image of one stain in each channel. One channel was used to identify all components in the tissue counterstained with methyl green (*i.e.*, all nuclear components), and the other channel was used to calculate the density of brown stain (diaminobenzidine) per tissue area to identify the proportion of cells stained with antibodies to TSP-1. Image analysis-based units of staining are reported as absorbance (*A*). A negative control accompanying each specimen was used to set the antibody threshold such that nonspecific background staining was eliminated from the study measurement. A minimum of 10 fields with varying intensities were examined for all radical prostatectomy, transurethral prostatectomy, or biopsy specimens.

Statistical Analysis. HScore results were correlated with Gleason score, stage, and survival. Disease-specific survival was calculated from the date of tissue diagnosis to the date of death attributable to prostate cancer or the date of the last follow-up, by the Kaplan-Meier method, and survival of the two subgroups was compared by the log-rank test (15). Deaths attributable to unknown events were considered an event for the analysis presented, although a second analysis performed with censoring of unknown deaths showed similar statistical signif-

Table 1 AI[a]

Score	mp53 HScore	TSP-1 OD	Vessel counts per ×200 Field
1	0–29	≥30	0–29
0	30–59	25–29	30–69
−1	60–89	20–24	70–84
−2	90–119	15–19	85–99
−3	120–149	10–14	100–122
−4	≥150	0–9	≥123

[a] Overall, AI was derived from the sum of scores (+1 to −4) corresponding to the mp53 HScore, TSP-1 absorbance and vessel counts. For example, a case expressing an mp53 HScore of 90, a TSP-1 absorbance of 10, and a vessel count of 85 would have an overall AI value of −7 (−2 plus −3 plus −2).

icance (data not shown). Fleming-Herrington estimates of survival for "the average patient" in this series were applied to the impact of AI on survival. χ^2 and Spearman correlation tests examined the strength of association between angiogenesis and TSP-1 and p53. The Cochran-Armitage test for linear trend was applied to determine whether AI was related to increasing Gleason score. All tests for significance were two-tailed.

AI. The positive correlation between mp53 and angiogenesis and the inverse association between mp53 and TSP-1 determined by Spearman correlation coefficient prompted the generation of an AI by integrating these three factors for each case (9, 10). Biomarker IHC or image analysis scores were assumed to be continuous variables with stepwise progression. A biomarker specific AI score was created from the original IHC scores, image analysis scores, and CD31-based microvessel counts by assigning integer values to a specified range of scores for each biomarker. In a previous study by our group, breast cancer patients whose specimens had HScores ≥30 for mp53 demonstrated poorer survival compared with patients with tumor mp53 HScores <30 (10). Hence, the lowest dichotomous cutpoint at which the biomarker showed a statistical association with survival was determined to be 30 for mp53 HScores and was therefore assigned an AI score of " 0." Given that the mp53 HScores ranged from 0 to >150, mp53-specific HScores were then categorized into six equal domains, with p53 HScores <30 defined as +1, indicating a favorable association with improved survival outcomes. The remainder of the biomarker HScore ranges that were associated with poor survival outcomes were assigned AI scores of −1 to −4, corresponding to increasing mp53 HScores, with −4 assigned to HScores ≥150. TSP-1 absorbance units and vessel counts were assigned biomarker-specific scores between +1 and −4 in a similar manner. The AI value was derived for each specimen by summing the biomarker-specific scores for p53, TSP-1, and CD31, as shown in Table 1. Favorable AI scores were assigned positive numbers, with a maximum of +3, whereas more adverse AI scores were negative, with a minimum of −12. This AI scoring system was initially applied to breast cancer (test set) and was subsequently applied to the present cohort of prostate cancer patients without modification (study set; Ref. 10).

RESULTS

Representative photomicrographs of immunohistochemical staining of p53, TSP-1, and microvessel density (CD31) are shown in Fig. 1. When p53, TSP-1, and microvessel density (CD31) were measured in the same prostate cancer specimens, a highly significant association was found between acquisition of mp53 and increased angiogenesis ($P < 0.0001$). There was also an inverse relationship between mp53 and TSP-1 absorbance units ($P = 0.002$) and between TSP-1 and angiogenesis ($P < 0.0001$), as depicted in Table 2.

These associations were similar to those observed previously in melanoma and breast cancer, and their integration into an AI was performed and applied to the cases of prostate cancer studied here (9, 10). The results of Fisher's exact test for association between AI and clinical variables are presented in Table 3. No statistically significant difference in age, stage, or radiation and hormone treatment was found when patient characteristics were compared between the two groups stratified by AI ≤ −6 ($n = 18$) and AI ≥ −5 ($n = 80$). The statistically significant associations were between AI and Gleason score ($P = 0.0003$), TSP-1 ($P = 0.003$), CD31 ($P = 0.014$), and p53 ($P = 0.0001$).

As shown in Table 4, univariate analysis demonstrated that patient age ($P < .0007$), stage (3–4 *versus* 0–2; $P = 0.0003$), Gleason score (≥7 *versus* 2–6; $P = 0.005$), mp53 (≥60 *versus* <60; $P = 0.003$), CD31-based microvessel counts (≥65 *versus* <65; $P < 0.017$), and AI (≤ −6 *versus* ≥ −5; $P = 0.002$) were significantly associated with patient survival. As expected, hormonal treatment status was significantly associated with poor survival ($P = 0.0001$), because this form of treatment is usually given to patients with advanced stage of disease. TSP-1 and prostatic bed radiation treatment status were not significant in univariate analysis. p53 and microvessel counts individually were not statistically significant prognostic factors after adjustment for age, stage, Gleason score, and hormonal treatment in multivariate analysis (data not shown).

Patients with an AI of −5 or greater showed significantly longer disease-specific survival ($P = 0.002$) compared with patients with AI of −6 or less in univariate analysis (Fig. 2*A*). In addition, multivariate analysis showed the AI to be independently associated with survival ($P < .005$) after adjustment for age and hormonal treatment status (Fig. 2*B*). A 3.2-fold increase in mortality was noted for patients with an AI of −6 or less, compared with those with an AI of −5 or greater in multivariate analyses (Table 5). After adjusting for hormonal treatment status, both stage and Gleason score lost significance, because stage and Gleason score dictated treatment, whereas AI remained an independent predictor of disease-specific survival.

We noted a significant association between an increasing Gleason score and an increasing percentage of patients having mutant p53, lower TSP-1, higher CD31, and an AI less than or equal to −6. This was demonstrated by Cochran's test for linear trend (Table 6). The association between stage, CD-31, and TSP-1 demonstrated significance, whereas associations between stage, mp53, and AI did not reach statistical significance by Cochran's test for linear trend (Table 7).

DISCUSSION

This study of 98 prostate cancer specimens revealed a correlation between IHC-based detection of p53 protein, decreased levels of the angiogenesis inhibitor TSP-1, and in-

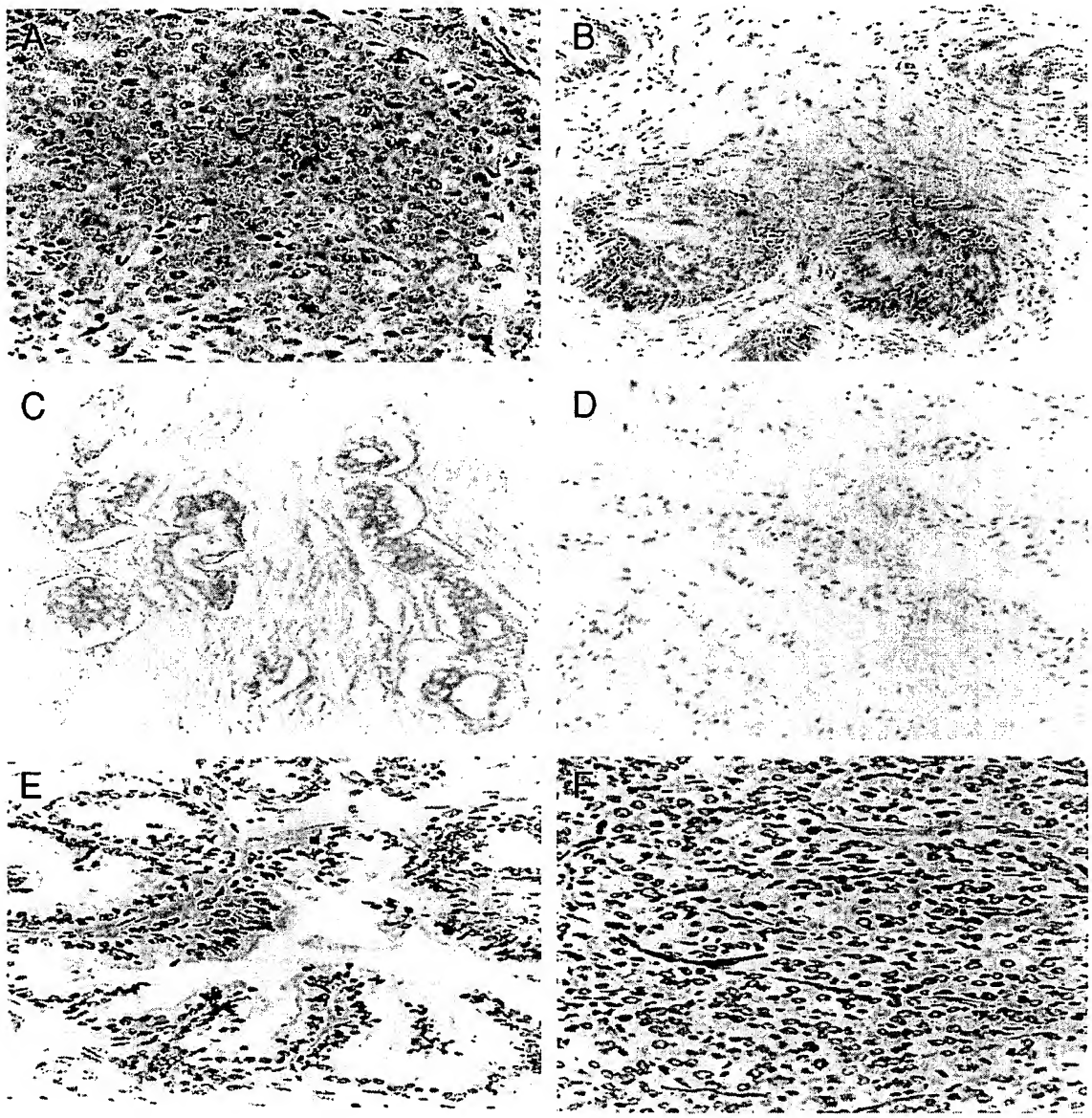

Fig. 1 Photomicrographs depicting representative IHC staining patterns for prostate carcinoma. *A*, mp53 negative; *B*, mp53 positive; *C*, high TSP-1; *D*, low TSP-1; *E*, low CD31; *F*, high CD31. ×200.

Table 2 Association between TSP-1, mp53, and vessel counts

	Correlation coefficient[a]	*P*
TSP-1 *vs.* Vessel Counts	0.465	<0.0001
mp53 *vs.* Vessel Counts	0.533	<0.0001
TSP-1 *vs.* mp53	−0.302	<0.002

[a] Spearman correlation.

creased angiogenesis. Furthermore, integrating IHC staining results for mp53, TSP-1, and vessel counts into an AI resulted in a scoring system strongly associated with Gleason score and with prostate cancer patient survival. Our current study applied the AI scoring system validated previously using breast cancer patients to prostate cancer patients (9). In this study, we found that patients with AI index scores ≤ -6 compared with patients with AI index scores ≥ -5 demonstrated poor survival in both univariate and multivariate analyses. These data support the concept that integrating multiple markers related to the regulation of angiogenesis may be a more robust predictor of clinical outcome than when single biomarkers are applied to determine prognosis (9, 10, 13).

Angiogenesis has been shown to be associated with poor prognosis in many tumor types, including prostate and breast carcinomas (16–18). Angiogenesis results from disruption of the homeostatic balance between factors that stimulate endothelial cell proliferation and those that inhibit neovascular formation. The relationship between the loss of p53 function, decreased expression of TSP-1, and increased angiogenesis was initially demonstrated by Bouk *et al.* (19), who studied Li-

Table 3 Patient characteristics stratified by AI

Patient characteristics	Overall *n*	AI ≥ −5		A to AI		Fisher's exact *P*
		n	%	*n*	%	
Age						
≤70	51	42	56	9	56	1.00
>70	40	33	44	7	44	
Stage						
≤2	62	52	66	10	63	0.778
≥3	33	27	34	6	37	
Gleason score						
≤6	60	56	70	4	22	0.0003
≥7	38	24	30	14	78	
mp53 category						
<60	84	79	99	5	28	0.0001
≥60	14	1	1	13	72	
TSP-1 category						
<20	72	54	68	18	100	0.003
≥20	26	26	32	0	0	
Vessel counts						
<65	48	45	56	3	17	0.004
≥65	50	35	44	15	83	
Hormone therapy						
No	62	53	70	9	56	0.380
Yes	30	23	30	7	44	
Radiation therapy						
No	71	57	76	14	87	0.337
Yes	20	18	24	2	13	
Surgery						
TURP[a]	43	35	47	8	50	0.549
Radical prostatectomy	41	36[b]	48	5	31	
Incisional biopsy[c]	7	4[d]	5	3	19	

[a] Sixteen patients had prior hormonal treatment, 8 patients had prior radiation, and 4 patients had prior hormonal and radiation treatment, whereas 15 patients had no prior treatment.
[b] Followed by radiation therapy in 6 patients.
[c] Followed by hormonal therapy.
[d] Followed by radiation therapy in 2 patients.

Table 4 Univariate analysis of various prognostic features

Prognostic features	*n*	Comparison of survival curves[a]
Age (≤69 *vs.* ≥70)	91	0.0324
Stage (0–2 *vs.* 3–4)	95	0.0003
TSP-1 (≥20 *vs.* ≤20)	98	0.2243
Vessel counts (<65 *vs.* ≥65)	98	0.0169
mp53 (<60 *vs.* ≥60)	98	0.0034
Gleason score (<7 *vs.* ≥7)	98	0.0053
AI (≥−5 *vs.* ≤−6)	98	0.0022
Radiation treatment (Yes *vs.* No)	91	0.7722
Hormone treatment (Yes *vs.* No)	92	0.0001

[a] *P* determined by the log-rank test.

Fraumeni fibroblasts carrying p53 mutations. Bouk's group was able to demonstrate that mutation in the *p53* gene resulted in the translation of a defective p53 protein that was unable to up-regulate TSP-1, resulting in a proangiogenic state. Transfecting wt p53 into defective cells resulted in increased TSP-1 production and restoration of the antiangiogenesis state associated with wt p53. Further, Guo *et al.* (20) have demonstrated that TSP-1 induces apoptosis of endothelial cells, defining one mechanism of its antiangiogenesis effects. On the basis of these observations, we evaluated whether there was a relationship between p53, TSP-1, and angiogenesis in malignant tissues. We subsequently noted that these factors were interrelated in melanoma and breast carcinoma specimens (9, 10). Others have recently reported similar associations in bladder and colorectal carcinoma (8, 21, 22). In contrast, in a small series, Kawahara *et al.* (23) were unable to find a relationship between p53 and TSP-1 in cholangiocarcinoma or hepatocellular carcinoma. These results can be explained, in part, by the fact that TSP-1 expression may be influenced by c-*jun* or other factors independent of p53 (24, 25). Hsu *et al.* (26) have demonstrated that loss of an allele on chromosome 10 may mediate loss of TSP-1 up-regulation independent of p53 in human glioblastomas. Similarly, Fontanini *et al.* (27) examined the relationship between mutations in p53 and TSP-1 cDNA copy numbers in 19 cases of non-small cell lung cancer, finding no relationship between p53 and TSP-1. However, they did note an inverse association between TSP-1 cDNA and fibroblast growth factor protein levels (27). Bleuel *et al.* (28) have suggested that TSP-1 is a potential tumor suppressor gene based on their observation that *TSP-1* gene deletion secondary to loss of chromosome 15 was associated with the development of a highly vascularized tumor phenotype that could be reversed by TSP-1 transfection. These studies indicate that TSP-1 regulation is multifactorial, and that it may differ, in part, depending on the tissue under investigation. Data presented in this paper suggest that TSP-1 is significantly asso-

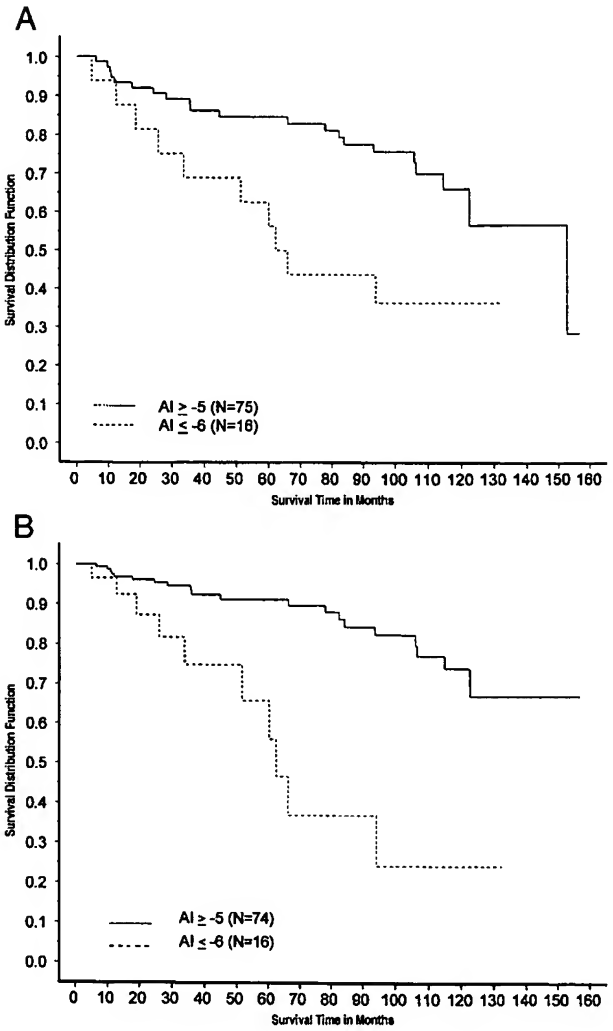

Fig. 2 *A*, Kaplan-Meier plot of disease-specific survival comparing of AI ≥ -5 *versus* AI ≤ -6. No adjustment of covariates was made (n = 91). *B*, comparison of disease-specific survival probability for cases with AI ≥ -5 *versus* AI ≤ -6 adjusted for covariates using the Fleming-Herrington estimate of survival adjusted for age and hormonal treatment status (n = 90).

Table 5 Multivariate analysis of survival with AI[a]

Prognostic features	χ^2 P	Risk ratio	95% CI[b]
Hormone treatment (Yes *vs.* No)	0.0001	8.058	3.585–18.116
AI (≤ -6 *vs.* ≥ -5)	0.005	3.237	1.435–7.302
Age	0.016	1.052	1.010–1.097

[a] n = 90.
[b] CI, confidence interval.

Table 6 Marker profile of prostate cancer progression (Gleason scores 2–3 *vs.* 4–6 *vs.* ≥7)

	Gleason score			
Marker status	2–3 (n = 10) (10%)	4–6 (n = 50) (51%)	≥7 (n = 38) (39%)	P[a]
TSP-1				
≤20	3	32	37	0.001
≥20	7	18	1	
Vessel counts				
<65	10	30	6	0.001
≥65	0	20	32	
mp53 HScore				
<60	10	47	27	
≥60	0	3	11	0.002
AI				
≤−6	0	4	14	
≥−5	10	46	24	0.001

[a] Cochran-Armitage test for trend.

ciated with both p53 status and microvessel density in prostate carcinoma.

Many investigators who have evaluated the prognostic significance of biomarkers in prostate cancer patients have suggested that p53 and angiogenesis are relevant to outcomes. Theodorescu *et al.* (6) found that mp53 and Rb reactivity were independently predictive of disease-specific survival in 71 patients who underwent radical prostatectomy for stage A1 to B2 prostate carcinoma. Moreover, these two factors were stronger predictors of survival than conventional pathological parameters. Similarly, Silberman *et al.* (29) found a correlation between angiogenesis and progression after radical prostatectomy in patients with Gleason scores of 5 to 7. Bettencourt *et al.* (30) used a monoclonal antibody directed against CD34 to determine angiogenesis levels to analyze the link between microvessel density and recurrence in 149 patients who had undergone radical prostatectomy. They found that the rate of recurrence-free survival for 5 years was 71% for patients with counts <90 vessels/microfield and 51% for patients with counts >90. Offersen *et al.* (31) used factor VIII staining of prostate carcinoma specimens to determine whether maximal microvessel density was associated with outcomes. They found that the maximal microvessel density was significantly associated with survival in both univariate and multivariate analyses. Taken together, these studies suggest that levels of angiogenesis may provide prognostic information about clinical outcomes in prostate cancer.

Our results suggest that the AI, which reflects an angiogenic phenotype constructed from the immunostaining results of three different molecular factors influencing proliferation of new blood vessels, can serve as a more robust predictor of survival than individual prognostic markers. In this study, AI was more significant than stage and Gleason score as a predictor of survival, with a 3.2-fold difference in survival times between patients with AI of ≥ -5 and patients with AI ≤ -6.

Our study focused on a heterogeneous population of patients comprised of those with prostate-confined cancer undergoing prostatectomy as well as patients with metastatic disease. If the relevance of the AI is confirmed in a more homogeneous group of patients, it may be feasible to stratify patients to expectant *versus* active treatment at the time of diagnostic biopsy, based on their prognostic marker profile. Although our series of 98 blocks included only seven needle biopsy specimens, all seven were adequate for evaluation of the three biomarkers that comprised the AI. The simultaneous use of three

Table 7 Marker profile of prostate cancer progression (stages 0–2 *vs.* 3–4)

	Stage		
Markers studied	0–2 (n = 62)	3–4 (n = 33)	P^a
TSP-1			
>20	41	28	0.051
≤20	21	5	
Vessel counts			
<65	34	11	0.046
≥65	28	22	
mp53			
<60	55	29	0.904
≥60	7	4	
AI			
≤−6	10	6	0.799
≥−5	52	27	

[a] Cochran-Armitage test for trend.

biologically relevant factors integrated into the AI may partly address the issue of intratumoral heterogeneity of marker expression and should improve the predictive accuracy of prognostic testing. Because our study included only seven needle biopsy specimens, a larger study conducted to correlate clinical outcome with AI determined on pretherapy biopsy material would be an essential adjunct in assessing the role of AI in prostate cancer management. Furthermore, it will be important in future studies to evaluate the concordance of AI scoring in small pretherapy needle biopsy material with markers studied on subsequent prostatectomy specimens to quantify tumor heterogeneity.

Our retrospective study results presented here have defined a study set of HScores for mp53, TSP-1, and angiogenesis that comprise the AI. The simultaneous evaluation of molecular events that are associated with the angiogenic pathway may not only assist in defining the need for aggressive treatment *versus* observation but may eventually guide treatment with antiangiogenesis compounds. We plan to carry out prospective studies to confirm the value of the AI in prostate cancer management.

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Homeostatic Modulation of Cell Surface KDR and Flt1 Expression and Expression of the Vascular Endothelial Cell Growth Factor (VEGF) Receptor mRNAs by VEGF*

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Vascular endothelial cell growth factor (VEGF) is a potent angiogenic factor expressed during embryonic development, during wound healing, and in pathologies dependent on neovascularization, including cancer. Regulation of the receptor tyrosine kinases, KDR and Flt-1, to which VEGF binds on endothelial cells is incompletely understood. Chronic incubation with tumor-conditioned medium or VEGF diminished ^{125}I-VEGF binding to human umbilical vein endothelial cells, incorporation of ^{125}I-VEGF into covalent complexes with KDR and Flt1, and immunoreactive KDR in cell lysates. Receptor down-regulation desensitized VEGF activation of mitogen-activated protein kinase (extracellular signal-regulated kinases 1 and 2) and p38 mitogen-activated protein kinase. Preincubation with VEGF or tumor-conditioned medium down-regulated cell surface receptor expression but up-regulated KDR and Flt-1 mRNAs, an effect abrogated by a neutralizing VEGF antibody. Removal of VEGF from the medium led to recovery of ^{125}I-VEGF binding and resensitization of human umbilical vein endothelial cells. Recovery of receptor expression was inhibited by cycloheximide, indicating that augmented VEGF receptor mRNAs, and not receptor recycling from a cytoplasmic pool, restored responsiveness. As the VEGF receptors promote endothelial cell survival, proliferation, and other events necessary for angiogenesis, the noncoordinate regulation of VEGF receptor proteins and mRNAs suggests that human umbilical vein endothelial cells are protected against inappropriate or prolonged loss of VEGF receptors by a homeostatic mechanism important to endothelial cell function.

Angiogenesis is an important component of embryonic vascular development, wound healing, and organ regeneration as well as pathological processes such as diabetic retinopathies, rheumatoid arthritis, and tumor growth and spread (1, 2). A network of growth factors and cytokines regulate angiogenesis. Some of these, such as tumor necrosis factor, transforming growth factor, angiogenin, and prostaglandin E_2, induce angiogenesis indirectly (1, 2), whereas other factors that play a role in blood vessel development, such as the acidic and basic fibroblast growth factors and platelet-derived growth factor, are mitogens for many cell types.

Vascular endothelial cell growth factor (VEGF)[1] is unique, being an endothelial cell-specific mitogen that promotes the proliferation and movement of endothelial cells, remodeling of the extracellular matrix, formation of capillary tubules, and vascular leakage (3–8). VEGF is produced by normal and transformed cells (9, 10) and plays an obligate role in the development of the fetal cardiovascular system as well as a significant role in the physiology of normal vascular and in the progression of cancer (11–15). Interference of VEGF action *in vivo* by administration of a monoclonal antibody that neutralizes VEGF activity or through the introduction of dominant negative VEGF receptor constructs inhibits tumorigenesis in animal models of colon cancer or glioblastoma (16–18). Thus, it is of considerable importance to understand how the VEGF/VEGF receptor signaling system works and is regulated.

In the endothelium, VEGF exerts its actions by binding to two cell surface receptor tyrosine kinases, KDR (the human homolog of Flk1) and Flt1 (19–23). Flt1 binds VEGF with high affinity but is poorly expressed by endothelial cells, making it difficult to detect, whereas KDR binds VEGF with somewhat lower affinity but is the more highly expressed and readily detected receptor (24, 25). Mouse embryos deficient in Flk1 or Flt1 die between days 8.5 and 9.5, and the phenotypes of the knockout animals are distinct (26, 27). Endothelial and hematopoietic cell development are impaired in Flk1 null mice (26), whereas endothelial cells overgrow and blood vessels are disorganized in Flt1 null mice (27), indicating that the receptors have different functions. Consistent with this conclusion, VEGF mutants that selectively bind KDR/Flk1 induce endothelial cell proliferation and survival (28), whereas the ability of VEGF and placenta growth factor, a VEGF homolog that binds Flt1 but not KDR/Flk1, to induce chemotaxis and procoagulant activity associates these responses with signaling through Flt1 (29–31). Thus, the functions of VEGF are segregated between two structurally related receptors.

We previously demonstrated that VEGF promotes the tyrosine phosphorylation of a group of signaling molecules that contain SH2 domains and associated this process with endothelial cell proliferation (32). Subsequent studies with porcine

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[1] The abbreviations used are: VEGF, vascular endothelial cell growth factor; KDR/Flt1, kinase domain-containing receptor/fetal liver kinase; Flt1, *fms*-like tyrosine kinase; MAPK, mitogen-activated protein kinase; HUVEC, human umbilical vein endothelial cell(s); MEM, modified Eagle's medium; PBS, phosphate-buffered saline; bFGF, basic fibroblast growth factor; ERK, extracellular signal-regulated kinase.

aortic endothelial cells and NIH 3T3 cells overexpressing KDR/Flk1 or Flt1, bovine aortic endothelial cells, immortalized endothelial cells, and HUVEC have begun to identify the signaling proteins downstream of KDR/Flk1 or Flt1 (25, 33–41). Thus, considerable insight into the functions and mechanisms of action of the VEGF receptors has been achieved.

The present study was aimed at defining how expression of the VEGF receptors is regulated. We demonstrate that VEGF or tumor-conditioned medium down-regulates cell surface expression of KDR and Flt1 on HUVEC. Such down-regulation diminishes the ability of VEGF to stimulate MAPK (ERK1 and ERK2) and p38 MAPK activities. Whereas VEGF or tumor-conditioned medium down-regulated cell surface receptor expression, expression of the mRNAs for KDR and Flt1 was increased by VEGF or tumor-conditioned medium. Once VEGF or tumor-conditioned medium were no longer present, HUVEC rapidly recovered receptor expression and responsiveness to VEGF, a process of resensitization that was fully dependent on protein synthesis. The demonstration that loss of cell surface VEGF receptor expression and responsiveness to VEGF occur coincidentally with up-regulation of the VEGF receptor mRNAs indicates the operation of a homeostatic mechanism that replenishes cell surface receptor expression and restores HUVEC responsiveness once exogenous levels of VEGF are diminished.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human VEGF was a gift from Genentech, Inc. (South San Francisco, CA). Epidermal growth factor, bovine brain extract, and hydrocortisone were from Clonetics, Inc. (San Diego, CA). F-12, MEM, and MCDB 131 medium were from Life Technologies, Inc.

Cell Culture—The HeLa line of human cervical carcinoma cells was obtained from the ATCC (Manassas, VA); HM-7 and LS LiM6 human colon adenocarcinoma cells were kindly provided by Dr. Young Kim (University of California at San Francisco) (17). Cells were grown and maintained in a 1:1 mixture of F-12 and MEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin in a humidified incubator under 5% CO_2 at 37 °C. To isolate tumor-conditioned medium, confluent tumor cells were washed and then incubated in serum-free F-12/MEM (1:1) for 72 h. The conditioned medium was recovered from the cultures, centrifuged to remove cell debris, and stored at −70 °C. To test the effects of conditioned medium, cells were grown in equal volumes of conditioned medium and F-12/MEM.

HUVEC isolated according to the method of Jaffe *et al.* (42) were grown on rat tail collagen type I (100 μg/ml)-coated tissue culture plates in MCDB 131 medium supplemented with 10% FBS, 12 μg/ml bovine brain extract, 10 ng/ml epidermal growth factor, and 1 μg/ml hydrocortisone in a humidified incubator under 5% CO_2. Before exposure to VEGF or tumor-conditioned medium, HUVEC (passage 4–8) were washed with PBS and incubated with serum-free F-12/MEM for 8 h. The HUVEC were then incubated in serum-free F-12/MEM supplemented with human recombinant $VEGF_{165}$ or a 1:1 mixture of tumor-conditioned medium and serum-free F-12/MEM.

Binding of ^{125}I-VEGF to Receptors on the Surface of HUVEC—Human recombinant $VEGF_{165}$ was coupled to ^{125}I in an IODO-GEN-catalyzed reaction (43). Confluent HUVEC in 12- or 24-well plates were washed, incubated with serum-free F-12/MEM for 8 h at 37 °C, and then treated with VEGF or tumor-conditioned medium as described in the figure legends. After treatments, cell surface bound VEGF was dissociated from the HUVEC during three wash cycles with acidic PBS (pH 3) at 4 °C, followed by an additional wash with serum-free F-12/MEM. Control or treated HUVEC in ice-cold PBS, 0.2% bovine serum albumin were incubated with ^{125}I-VEGF in the absence or presence of a 200-fold excess of VEGF for 3 h at 4 °C. The cells were washed three times with ice-cold PBS and lysed into 0.5% SDS, 0.2 N NaOH, and ^{125}I was assayed in a γ-counter (Beckman). Specific binding, the difference in radioactive uptake in the absence and presence of unlabeled VEGF, is normalized to the number of HUVEC in each tissue culture well.

Affinity Labeling of VEGF Receptors—Confluent HUVEC in 60-mm dishes were washed, incubated in serum-free F-12/MEM for 8 h, and then treated with VEGF (20 ng/ml) or tumor-conditioned medium at 37 °C for 24 h. The cells were subjected to three cycles of acidic wash conditions (PBS, pH 3.0, 4 °C) and then to a final wash with serum-free F-12/MEM before incubation with 200 pM ^{125}I-VEGF in PBS, 0.2% bovine serum albumin for 2 h at 4 °C. The cells were washed twice with ice-cold PBS, 0.1% bovine serum albumin (pH 7.4) before ^{125}I-VEGF was covalently coupled to cell surface VEGF receptors by incubation with the organic cross-linking reagent disuccinimidyl suberate for 15 min at 4 °C (36). The cross-linking reaction was quenched by the addition of 10 mM Tris-HCl, pH 7.5, 200 mM glycine and 2 mM EDTA, and the cells were washed four times with ice-cold PBS and scraped from the culture dishes. After centrifugation at 12,000 rpm for 5 min, the cell pellets were lysed by incubation in 100 μl of radioimmune precipitation assay lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 5 μg/ml leupeptin) for 15 min at 4 °C, after which insoluble debris was removed by centrifugation. Equal amounts of protein from each lysate were fractionated on 7.5% polyacrylamide gels, and affinity labeled complexes were detected by autoradiography.

Western Blotting—Confluent HUVEC in 10-cm tissue culture plates were starved for 8 h in serum-free F-12 MEM medium and then incubated with tumor-conditioned medium or VEGF for 24 h. The cells were exhaustively washed with serum-free medium, scraped from the culture plates, briefly centrifuged, and then solubilized into radioimmune precipitation assay lysis buffer as described above. Equal amounts of protein from the lysates were fractionated on 10% polyacrylamide gels and then transferred to Hybond ECL Nitrocellulose membranes (Amersham Pharmacia Biotech). The Western blots were probed with an antibody directed against KDR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then incubated with a donkey anti-rabbit antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). The SuperSignal chemiluminescent detection system (Pierce) was used for protein detection.

Mitogen-activated Protein Kinase (MAPK) and p38 MAPK Activities—Confluent HUVEC in six-well tissue culture plates were serum-starved for 8 h at 37 °C and then exposed to VEGF as described in the legend to Fig. 3. The cells were washed with PBS and lysed into 200 μl of lysis buffer (1:1 mixture of radioimmune precipitation assay buffer and 2× Laemmli buffer). The lysates were boiled at 100 °C for 3 min and centrifuged at 12,000 × *g* (4 °C, 5 min) to remove debris. Equal amounts of protein were fractionated on 10% polyacrylamide gels and transferred to Hybond ECL nitrocellulose membranes, which were probed with antibodies that recognize dually phosphorylated (activated) MAPK (Promega Inc., Madison, WI) or p38 MAPK (New England Biolabs). The blot was then incubated with anti-rabbit secondary antibodies coupled to horseradish peroxidase, and the SuperSignal detection kit (Pierce) was used for protein detection.

Northern Blot Analysis of VEGF Receptor Expression—Confluent, serum-starved HUVEC were incubated with VEGF or tumor-conditioned medium for various times. Total RNA was isolated using the TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the procedure recommended by the manufacturer. 10 μg of total RNA was fractionated on a formaldehyde-agarose gel and transferred to Hybond N nylon membranes. Specific 510-base pair Flt-1 and 1,100-base pair KDR cDNA probes were generated by reverse transcription-polymerase chain reaction and cloned into the pT Adv plasmid (CLONTECH, California). The sequences of the cDNA probes were verified by sequencing using Thermo Sequenase (Amersham Pharmacia Biotech). The 18 S ribosome RNA cDNA probe was a gift from Dr. Yuping Yang (Department of Pediatric Cardiology, University of California, San Francisco). The probes were labeled with [^{32}P]CTP using a random primer DNA labeling kit (Amersham Pharmacia Biotech) and then used to probe the Northern blots. After autoradiography, films were scanned with a GS-700 imaging densitometer (Bio-Rad, Hercules, CA). The expression of KDR and Flt-1 mRNA is normalized to the level of 18 S RNA for each lane of the blot.

Reproducibility of Data—Each experiment has been repeated at least three times with consistent results.

RESULTS

VEGF and Tumor-conditioned Medium Down-regulate Cell Surface VEGF Receptor Expression—To determine the effect of tumor-conditioned medium or VEGF on the expression of cell surface VEGF receptors, HUVEC were incubated with medium conditioned by HeLa or LS LiM6 cells or various concentrations of VEGF. The cells were then subjected to acidic washes to promote ligand dissociation, rendering cell surface VEGF receptors that had not been internalized available for interaction with subsequently applied ^{125}I-VEGF. As illustrated by the data in Fig. 1*A*, pretreatment of HUVEC with tumor-condi-

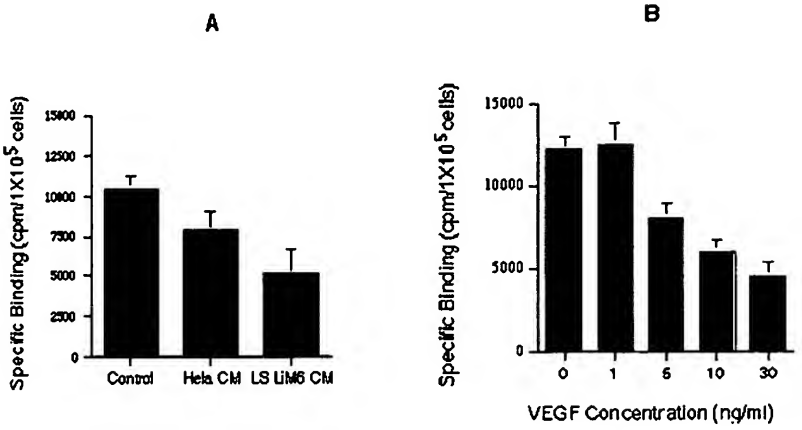

FIG. 1. **Effect of VEGF and tumor-conditioned medium on VEGF receptor expression.** Serum-starved HUVEC were incubated with tumor-conditioned medium or VEGF for 24 h at 37 °C before being washed under acidic conditions to dissociate residual VEGF bound to the cell surface. The specific binding of ^{125}I-VEGF to equal numbers of HUVEC treated with medium conditioned by HeLa or LS LiM6 cells (*A*) or various concentrations of VEGF (*B*) was then assayed. Each *bar* represents the mean of triplicate determinations ± S.D.

tioned medium diminished the subsequent binding of ^{125}I-VEGF 25 and 52%, respectively. Furthermore, 30 ng/ml VEGF diminished the subsequent binding of ^{125}I-VEGF by 63% (Fig. 1*B*), showing that VEGF down-regulates its own receptors and does so in a dose-dependent manner.

Affinity labeling assays also evaluated the effect of tumor-conditioned medium and VEGF on cell surface receptor expression. By using disuccinimydyl suberate, an organic cross-linking reagent, we covalently coupled ^{125}I-VEGF to VEGF receptors on HUVEC, leading to the formation of a predominant complex of 245 kDa and a less abundant 195-kDa complex (Fig. 2). By subtracting the contribution of ^{125}I-VEGF (monomeric molecular weight 14,000) to these complexes, it was determined that VEGF bound to species of 231 and 181 kDa, which correspond with the M_r values of KDR and Flt1, respectively. Analysis by densitometry revealed that pretreatment of HUVEC with HeLa and LS LiM6 conditioned medium inhibited the subsequent incorporation of ^{125}I-VEGF into the putative KDR complex by 32 and 70%, respectively, whereas 20 ng/ml VEGF decreased complex formation by 66% (Fig. 2). Such treatments produced a similar effect on the amount of Flt1 available for complex formation with ^{125}I-VEGF. The diminished incorporation of ^{125}I-VEGF into affinity-labeled complexes induced by tumor-conditioned medium or VEGF was comparable with the degree to which these agents diminished the specific binding of ^{125}I-VEGF to HUVEC (Fig. 1).

Western blot analysis also evaluated the effect of VEGF or tumor-conditioned medium on cell surface VEGF receptor expression. In this procedure, lysates of control HUVEC or of HUVEC pretreated with tumor-conditioned medium or two different concentrations of VEGF were fractionated by SDS-PAGE and then transferred to nitrocellulose membranes, which were probed with an antibody directed against KDR (Fig. 3). Under basal conditions, HUVEC expressed low levels of the unglycosylated 150-kDa and higher levels of an intermediate 200-kDa form of KDR. The mature 230-kDa form of KDR was also detected at a level of expression between that of the unglycosylated and intermediate receptor forms (44). The molecular mass of the mature form of the receptor was similar to that of the KDR within the affinity-labeled complex described above (Fig. 2). Expression of the immature and mature forms of KDR was down-regulated by tumor-conditioned medium or VEGF, the latter in a dose-dependent manner (Fig. 3). We could not successfully probe Flt1 expression by Western blotting, as an antibody with the requisite sensitivity and specificity for such analysis is not available. Our results show that VEGF receptor expression is regulated by ligand-induced down-regulation.

Effect of VEGF on MAPK Activity—We have demonstrated that KDR transduces the VEGF signal that leads to activation

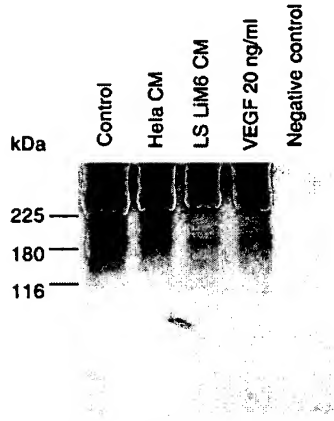

FIG. 2. **Affinity labeling of VEGF receptors.** Serum-starved HUVEC were treated with medium conditioned by HeLa or LS LiM6 cells or VEGF (20 ng/ml) for 24 h at 37 °C before dissociation of residually bound VEGF by acidic wash conditions. ^{125}I-VEGF was then covalently coupled to cell surface receptors, and the affinity-labeled complexes were fractionated on 7.5% polyacrylamide gels and detected by autoradiography. The control was without treatment, and negative control was with a 200-fold excess of cold VEGF when the cells were incubated with ^{125}I-VEGF.

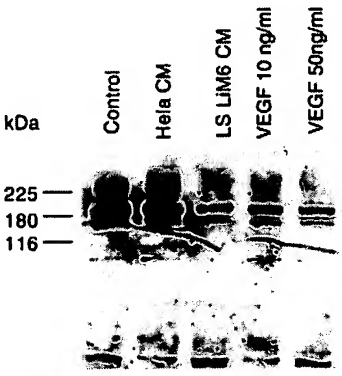

FIG. 3. **Western blot analysis of KDR expression.** HUVEC were treated with VEGF or tumor-conditioned medium for 24 h at 37 °C. Proteins in cell lysates were then fractionated on 7.5% polyacrylamide gels and transferred to nitrocelluose membranes. The Western blot was probed with an antibody against KDR.

of MAPK (40). This led us to conduct experiments to determine whether the down-regulation of KDR, demonstrated above, was associated with desensitization of the ability of VEGF to promote MAPK activation in HUVEC previously exposed to tumor-conditioned medium or VEGF. To address this possibility experimentally, we first treated HUVEC with various concentration of VEGF for 10 min before proteins in cell lysates were fractionated by SDS-PAGE and transferred to nitrocellulose

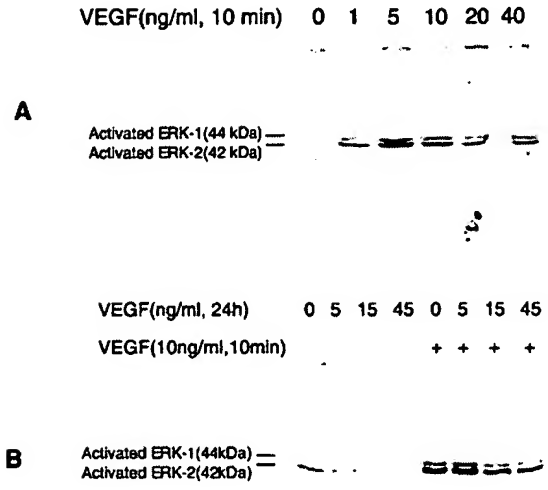

FIG. 4. **Desensitization of MAPK activation.** Serum-starved HUVEC were stimulated with VEGF for 10 min at 37 °C. Western blots of proteins fractionated from cell lysates were then hybridized with an antibody that recognizes dually phosphorylated (activated) MAPK (*A*) or treated with VEGF for 24 h at 37 °C and then rechallenged with medium or 10 ng/ml VEGF for 10 min at 37 °C before assay of MAPK activity by Western blot analysis (*B*).

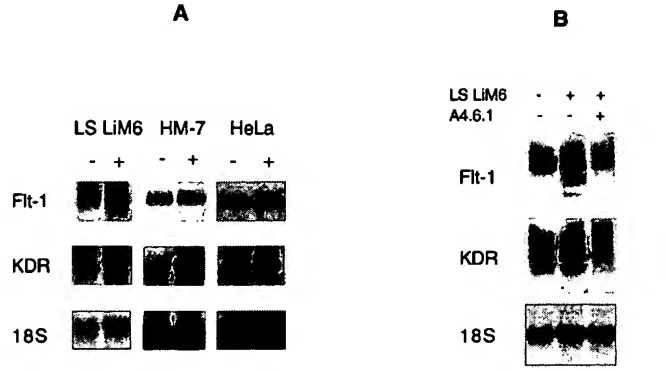

FIG. 5. **Effect of tumor-conditioned medium on Flt-1 and KDR mRNA expression.** Serum-starved HUVEC were cultured under tumor-conditioned medium for 24 h at 37 °C. Northern blots of total HUVEC RNA were hybridized with probes for KDR and Flt-1. *A*, KDR and Flt-1 mRNA expression in HUVEC treated with medium conditioned by LS LiM6, HM-7 and HeLa cells. *B*, KDR and Flt-1 mRNA expression in HUVEC incubated with medium conditioned by LS LiM6 cells in the absence or presence of a neutralizing, monoclonal antibody A4.6.1 to VEGF.

membranes, which were probed with the antibody that specifically recognizes dually phosphorylated (activated) MAPK. VEGF (1 ng/ml) increased the activity of both ERK1 and ERK2 in HUVEC. The stimulatory effect of VEGF on MAPK activation was maximal after exposure of HUVEC to 5–10 ng/ml VEGF, whereas higher mitogen concentrations elicited a diminished response. This latter effect may have been due to KDR down-regulation induced by exposure of HUVEC to the higher concentrations of VEGF (Fig. 4*A*).

Pretreatment of HUVEC with 0–45 ng/ml VEGF for 24 h diminished basal activity of MAPK in HUVEC in a dose-dependent manner. Furthermore, such pretreatment with 15 and 45 ng/ml VEGF diminished the ability of a second VEGF challenge to activate MAPK by 40 and 46%, respectively, when compared with cells not pretreated with VEGF (Fig. 4*B*). Thus, down-regulation of KDR resulted in a loss of HUVEC responsiveness to VEGF, as evidenced by desensitization of MAPK responsiveness to VEGF stimulation.

VEGF and Tumor-conditioned Medium Up-regulate Flt-1 and KDR mRNA Expression—Northern blot analysis was used to investigate the effect of tumor-conditioned medium and VEGF on the cellular content of the mRNAs for KDR and Flt-1. As illustrated by Fig. 5*A*, 24 h of exposure of HUVEC to medium conditioned by LS LiM6, HM-7, and HeLa cells increased the levels of Flt-1 mRNA by 2.5-, 1.7-, and 1.5-fold, respectively, whereas the level of KDR mRNA was increased 2.2-, 1.6-, and 1.5-fold. To determine whether the effect of tumor-conditioned medium was mediated by VEGF, tumor-conditioned medium was incubated for 2 h at room temperature with antiserum A4.6.1, which neutralizes VEGF activity; such treatment abrogated the ability of the conditioned medium to up-regulate the mRNAs for Flt-1 and KDR (Fig. 5*B*). The neutralizing antiserum to VEGF, by itself, also somewhat diminished the basal levels of Flt-1 and KDR mRNA expression. This observation may be explained by the ability of HUVEC to constitutively produce a low level of VEGF, which would be expected to sustain expression of the mRNAs for the VEGF receptors; an unrelated antibody (αgD#952; Genentech), which is not able to neutralize VEGF, produced no change in basal or VEGF-stimulated levels of the Flt-1 and KDR mRNAs (data not shown).

The time course over which VEGF induced up-regulation of the mRNAs for Flt-1 and KDR was investigated. As illustrated by the data in Fig. 6*A*, up-regulation was detected within 12 h of cellular exposure to VEGF and increased thereafter. Incubation with 20 ng/ml VEGF increased Flt-1 mRNA expression 1.3-, 1.6-, and 2.0-fold after 12, 24, and 40 h, respectively, whereas the levels of KDR mRNA increased 1.3-, 1.6-, and 1.8-fold over the same time course. The effects of VEGF on Flt-1 and KDR mRNA expression were also dependent on the concentration of VEGF to which HUVEC were exposed (Fig. 6*B*). Here, up-regulation of both mRNAs was detected after exposure of HUVEC to 10 ng/ml VEGF, and the mRNA levels increased progressively with exposure to higher (20 and 40 ng/ml) concentrations of VEGF.

Control experiments were conducted to demonstrate that the effects of VEGF on receptor modulation were specific. Thus, Northern blot analyses were conducted with human foreskin fibroblasts. These cells did not contain mRNAs for either KDR or Flt1 under basal conditions. Furthermore, exposure of these cells to VEGF did not result in the induction of mRNA for either of the VEGF receptors (data not shown).

Thus, our results show that on HUVEC VEGF has opposite effects on the expression of cell surface VEGF receptors and the mRNAs that encode these receptors: the former are down-regulated in response to exposure to VEGF, whereas the effect of VEGF on the latter is quite the opposite, resulting in up-regulation.

Recovery of Cell Surface Receptor Expression—The time course over which cell surface VEGF receptor expression could be recovered after down-regulation was defined. To accomplish this, HUVEC were pretreated with 20 ng/ml VEGF for 24 h and then subjected to serum-free medium washes to remove VEGF from medium. Subsequently, the specific binding of 10 or 400 pM ^{125}I-VEGF to the HUVEC was assayed over time. Within 2 h, half of the lost binding capacity of the HUVEC was recovered (Fig. 7, *A* and *B*). Within 5 h, more than ¾ of the initial cellular binding capacity had been recovered. The K_d with which Flt-1 binds VEGF has been estimated at 10–18 pM, whereas the K_d for binding to KDR is in the range of 400 pM (22, 23). Thus, binding assays conducted with 10 pM ^{125}I-VEGF would preferentially assay Flt1 recovery, whereas binding assays conducted with 200–500 pM ^{125}I-VEGF would assay for recovery of the more abundant VEGF receptor, KDR. The recovery of VEGF binding capacity assayed at very low and

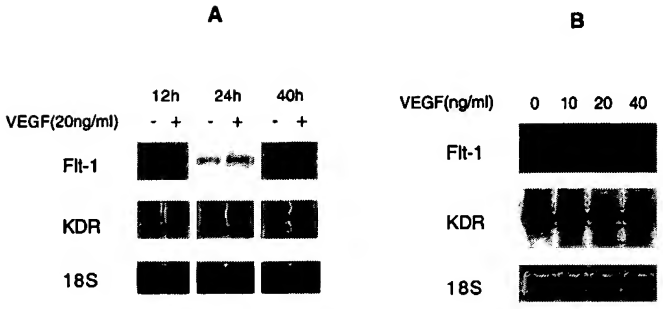

FIG. 6. **Effect of VEGF on KDR and Flt-1 mRNA expression.** Serum-starved HUVEC were incubated in the absence or presence of VEGF. Total RNA was isolated, and the expression of KDR and Flt-1 mRNAs was analyzed by Northern blot analysis. *A*, time course of the VEGF effect. *B*, dose response of the effect of VEGF after 24 h.

higher concentrations of ^{125}I-VEGF supports the conclusion that exposure of HUVEC to VEGF down-regulates both receptors, which are recovered after the agent inducing down-regulation is removed. Significantly, the protein synthesis inhibitor cycloheximide completely blocked receptor recovery, indicating that recycling from an internalized receptor pool or the presence of a preformed latent receptor population could not account for the recovery of VEGF receptor expression. Rather, such recovery is the result of new receptor synthesis from the up-regulated pools of KDR and Flt-1 mRNAs. Some loss of cell surface receptor expression was observed in HUVEC treated with cycloheximide. We speculate that this phenomenon results from continued turnover of cell surface receptors during a time when replenishment from the internal mRNA pools could not occur.

Resensitization of MAPK (ERK1 and ERK2) and p38 MAPK to Activation by VEGF—To determine whether recovery of cell surface receptor expression during up-regulation was accompanied by resensitization of HUVEC to VEGF, we assayed MAPK and p38 MAPK activities. The data presented in Figs. 8 and 9, respectively, show that the ability of VEGF to stimulate MAPK and p38 MAPK activation was diminished, relative to control, after cellular exposure to VEGF. However, once the exogenous VEGF used to desensitize the cells was removed, the responsiveness of HUVEC to VEGF was recovered in a time-dependent manner. Half-recovery of the ability of VEGF to activate MAPK was observed within 2 h, and full-recovery was detected within 6 h. A similar time course of recovery was observed for p38 MAPK. These time courses of resensitization coincide well with the time during which VEGF receptor expression at the surface of HUVEC was recovered (Fig. 7).

DISCUSSION

The temporal and spatial expression of VEGF and VEGF receptors in the embryo and female reproductive system provides evidence that VEGF is involved in developmental and hormonally regulated angiogenesis (1, 2, 13, 14, 23). Indeed, the unique importance of this angiogenic factor is illustrated by the observation that the loss of even a single allele for VEGF is lethal (45, 46). During wound repair, VEGF expression increases in the hyperproliferative endothelium, and defective expression of VEGF disorders this process (47). During tumorigenesis, transformed cells switch to an angiogenic phenotype characterized by down-regulation of inhibitors and up-regulation of inducers of angiogenesis (48). Among the most important, perhaps the preeminent, inducer of tumor angiogenesis is VEGF (2, 15–18). VEGF is also involved in the pathogenesis of psoriasis, rheumatoid arthritis, and proliferative retinopathies (49–51). Its role in so many important physiologic and pathologic conditions has prompted numerous studies aimed at defining how expression of VEGF is regulated. Most simply, and generally, augmented VEGF expression is induced by hypoxic conditions and by growth factors, such as IGF-1 (52–55).

As with VEGF, expression of the VEGF receptors is regulated. Flk1 is particularly abundant on the proliferating endothelial cells of vascular sprouts of embryonic and early postnatal brain; however, the level of Flk1 mRNA is dramatically reduced in adult brains in which endothelial cell proliferation has ceased (23). Augmented VEGF receptor expression occurs during wound healing (56), in ischemic eye disease (57), in the angiogenesis and stromal deposition associated with myocardial infarction (58), and in various cancers (17, 59–61). The conditions implicated in up-regulation of VEGF receptor expression appear the same as those responsible for up-regulation of VEGF. Thus, expression of the mRNAs for KDR and Flt1 is increased in lungs exposed to acute or chronic hypoxia (62). Gerber *et al.* (63) and Barleon *et al.* (64) found hypoxic induction of Flt1 mRNA expression in HUVEC; however, the former study assayed for but was unable to detect up-regulation of KDR. On the other hand, Waltenberger *et al.* (65) found that hypoxia induced KDR expression in HUVEC and in porcine aortic endothelial cells transfected so as to express KDR, which is not ordinarily present in such cells. Brogi *et al.* (54) have also documented up-regulation of KDR expression in HUVEC and microvascular endothelial cells exposed to medium conditioned by hypoxic myoblasts. A particularly interesting aspect of this investigation was the inability of various neutralizing antisera, including anti-VEGF, to abrogate KDR up-regulation, suggesting that an unidentified factor promoted this paracrine effect. Another report from Suzuki found that medium conditioned by hypoxic hepatoma cells was able to induce Flt-1 mRNA expression but not that of KDR in HUVEC (66). VEGF itself is reported to up-regulate KDR in cultures of cerebral slices, but not in HUVEC (67), whereas homologous up-regulation of both VEGF receptors has been found in bovine adrenal cortex endothelial cells (44). The dissimilarities among research reports may be ascribed to differences in the cells and experimental conditions used for experimentation and to the technical difficulty in detecting Flt1, which is poorly expressed in endothelial cells (24, 25). However, the conclusion that exposure of normal or neoplastic cells to hypoxia induces VEGF, which modulates expression of its own receptors, appears generally consistent with most studies performed to date.

In the present work, the effects of VEGF and tumor-conditioned medium on cell surface VEGF receptor expression as well as the mRNAs for the VEGF receptors were investigated. Whereas most previous studies have presented observations suggesting unrelenting up-regulation of the VEGF/VEGF receptor system under circumstances demanding an angiogenic response from a host organism, our work suggests the operation of a homeostatic mechanism that tightly regulates cellular responsiveness to VEGF.

Support for a homeostatic regulatory view for signaling through the VEGF/VEGF receptor signaling system is found in observations showing that exposure of cells to recombinant VEGF or VEGF in tumor-conditioned medium provokes loss of cell surface receptor expression. Down-regulation of Flt1 and KDR is demonstrated by assays quantitating the specific binding of high and low concentrations of ^{125}I-VEGF to HUVEC, affinity labeling, and, in the case of KDR (for which an adequate antibody is available), Western blot analysis. Functionally, such homologous receptor down-regulation desensitizes MAPK and p38 MAPK to activation by VEGF. Activation of MAPK and p38 MAPK are components of the mechanism through which VEGF induces endothelial proliferation (40) and migration (68, 69), respectively.

Once exogenous VEGF is removed, KDR and Flt1 are rapidly

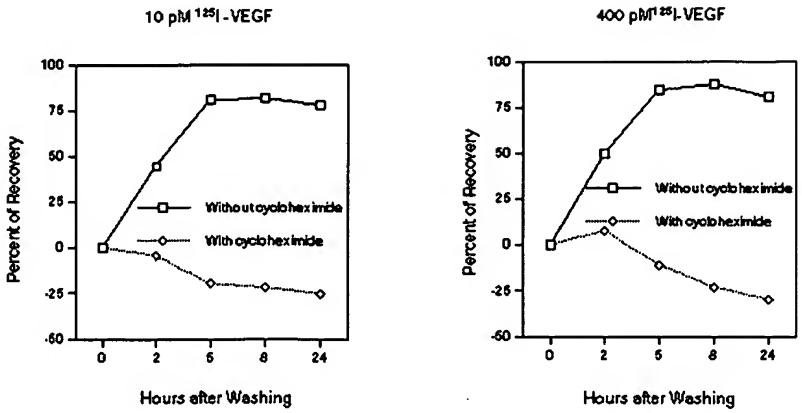

FIG. 7. **Recovery of VEGF cell surface receptor expression after chronic exposure of HUVEC to VEGF.** HUVEC were treated with 20 ng/ml VEGF for 24 h at 37 °C, subjected to serum-free medium washes, and cultured in MCDB131 medium containing 1% fetal bovine serum in the absence or presence of 0.1 mg/ml cycloheximide. At various times, the specific binding of 10 pM (*A*, *left*) or 400 pM (*B*, *right*) ^{125}I-VEGF to the cells was assayed after acidic washes.

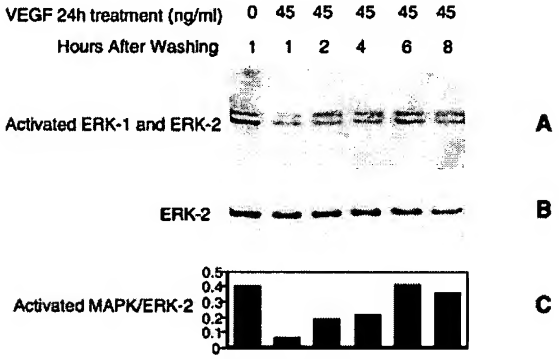

FIG. 8. **Recovery of MAPK responsiveness after down-regulation of VEGF receptor expression.** HUVEC were incubated with 0 or 45 ng/ml VEGF for 24 h at 37 °C. The cells were subjected to serum-free medium washes and then incubated in MCDB131 medium containing 1% fetal bovine serum for various times. *A*, a Western blot of proteins fractionated from cell lysates was probed with an antibody directed against dually phosphorylated MAPK (ERK1 and ERK2). *B*, to ensure that an equal amount of protein from lysates had been fractionated, the Western blot was stripped and reprobed with an antibody that recognizes ERK2. *C*, MAPK activity detected on the blot illustrated in *A* was quantitated by densitometry.

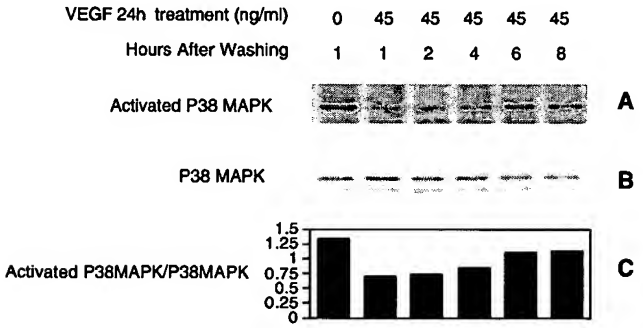

FIG. 9. **Recovery of p38 MAPK responsiveness after down-regulation of VEGF receptor expression.** HUVEC were treated as described in the legend to Fig. 8. *A*, a Western blot of proteins fractionated from cell lysates was probed with an antibody directed against activated p38 MAPK. *B*, to ensure that equal amounts of protein had been fractionated, the Western bot was reprobed with an antibody to p38 MAPK. *C*, p38 MAPK activity detected on the blot illustrated in *A* was quantitated by densitometry.

re-expressed at the surface of HUVEC. Recovery of receptor expression is complete within 3 h and reaches a level that closely approximates that present on the cell before exposure to VEGF. The recovery is not mediated by translocation of receptors from a preformed cytoplasmic pool, since it requires protein synthesis. Rather, down-regulation of cell surface VEGF receptor expression is accompanied by up-regulation of the mRNAs for KDR and Flt1. This augmented pool of mRNAs allows the rapid recovery of cell surface VEGF receptor expression once exogenous VEGF is removed from the HUVEC. Functionally, such up-regulation results in virtually completed recovery of HUVEC responsiveness to VEGF.

In addition to VEGF, a number of other peptide growth factors, including basic fibroblast growth factor (bFGF), hepatocyte growth factor, and transforming growth factor α, are believed to act as direct endothelial cell mitogens. bFGF is an angiogenic protein with potency comparable with that of VEGF (70). However, bFGF expression in primary human colorectal cancers is sparse, and immunoactivity resides principally in the stromal cells of these tumors (71). While bFGF lacks a signal sequence and is not likely to act as a carcinoma-derived endothelial growth factor in human colorectal cancer, bFGF can be detected in the extracellular matrix of many tissues and synergizes with VEGF in *in vitro* and *in vivo* assays of angiogenesis (72). Consequently, a contributory role of bFGF in colon cancer metastasis formation cannot be excluded, although it would seem unlikely to play a role in the assays described in the present report. Hepatocyte growth factor, also called scatter factor, stimulates endothelial cell proliferation and migration *in vitro* and is angiogenic in a rabbit cornea assay (73). The hepatocyte growth factor receptor, cMET, is overexpressed in colon cancers and may regulate the invasive behavior of these neoplasms (74). However, hepatocyte growth factor, the cMET ligand, appears to be expressed by stromal cells rather than the carcinoma cells in human colon cancer metastases.[2] While transforming growth factor α is secreted by a number of human colon cancer cell lines, this growth factor and its receptor are expressed at comparable levels in benign and malignant gastrointestinal epithelium (75).

VEGF is involved in wound repair, inflammation, and tumor growth, processes in which cytokines are elaborated. TNF and transforming growth factor β-1 modulate the expression of VEGF receptors (76–78), and TNF additionally inhibits VEGF activation of KDR and endothelial cell proliferative responses dependent on KDR by activating a protein-tyrosine phosphatase (79). However, the ability of a neutralizing antiserum to VEGF to block the ability of tumor-conditioned medium to induce up-regulation of the mRNAs for KDR or Flt1 suggests that VEGF is the predominant factor acting to modulate VEGF

[2] D. Wang, D. B. Donner, and R. S. Warren, unpublished observations.

receptor expression in the isolated cell cultures studied here.

VEGF is important to physiologic and pathologic processes dependent on neovascularization and is an endothelial cell survival factor (1, 2, 40, 80). Thus, it is reasonable to predict that HUVEC have mechanisms that would modulate VEGF responses, both down and up, through alterations of receptor expression. The present study demonstrates that down-regulation of receptor expression and desensitization of cellular responsiveness is coupled with up-regulation of receptor mRNA expression, which endows HUVEC with the ability to rapidly replenish the cell surface with VEGF receptors and fully recover responsiveness to VEGF.

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Design and Structure–Activity Relationship of a New Class of Potent VEGF Receptor Tyrosine Kinase Inhibitors

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A series of substituted 4-anilinoquinazolines and related compounds were synthesized as potential inhibitors of vascular endothelial growth factor (VEGF) receptor (Flt and KDR) tyrosine kinase activity. Enzyme screening indicated that a narrow structure–activity relationship (SAR) existed for the bicyclic ring system, with quinazolines, quinolines, and cinnolines having activity and with quinazolines and quinolines generally being preferred. Substitution of the aniline was investigated and clearly indicated that small lipophilic substituents such as halogens or methyl were preferred at the C-4′ position. Small substituents such as hydrogen and fluorine are preferred at the C-2′ position. Introduction of a hydroxyl group at the meta position of the aniline produced the most potent inhibitors of Flt and KDR tyrosine kinases activity with IC_{50} values in the nanomolar range (e.g. **10**, **12**, **13**, **16**, and **18**). Investigation of the quinazoline C-6 and C-7 positions indicates that a large range of substituents are tolerated at C-7, whereas variation at the C-6 is more restricted. At C-7, neutral, basic, and heteroaromatic side chains led to very potent compounds, as illustrated by the methoxyethoxy derivative **13** (IC_{50} < 2 nM). Our inhibitors proved to be very selective inhibitors of Flt and KDR tyrosine kinase activity when compared to that associated with the FGF receptor (50- to 3800-fold). Observed enzyme profiles translated well with respect to potency and selectivity for inhibition of growth factor stimulated proliferation of human umbilical vein endothelial cells (HUVECs). Oral administration of selected compounds to mice produced total plasma levels 6 h after dosing of between 3 and 49 μM. In vivo efficacy was demonstrated in a rat uterine oedema assay where significant activity was achieved at 60 mg/kg with the meta hydroxy anilinoquinazoline **10**. Inhibition of growth of human tumors in athymic mice has also been demonstrated: compound **34** inhibited the growth of established Calu-6 lung carcinoma xenograft by 75% (P < 0.001, one tailed t-test) following daily oral administration of 100 mg/kg for 21 days.

Introduction

Pathological angiogenesis has been associated with a variety of disease states including diabetic retinopathy, psoriasis, cancer, rheumatoid arthritis, atheroma, Kaposi's sarcoma, and haemangioma.[1,2] Altered vascular permeability is also thought to play a role in such processes.[3,4,5]

Several polypeptides with in vitro endothelial cell growth promoting activity have been identified, including acidic and basic fibroblast growth factors (aFGF bFGF) and vascular endothelial growth factor (VEGF). By virtue of the restricted expression of its receptors, the growth factor activity of VEGF, in contrast to that of the FGFs, is relatively specific toward endothelial cells. Recent evidence indicates that VEGF is an important stimulator of both normal and pathological angiogenesis.[6–10] VEGF has been shown to be secreted by human tumor cell lines in culture (e.g. glioma[11] and melanoma[12]). Moreover VEGF protein as well as mRNA for the VEGF receptors Flk-1/KDR have been identified in primary tumors of breast,[13,14] colon,[15,16] and renal origin.[17]

Receptor tyrosine kinases (RTKs) have been shown to be important mediators of signal transduction in cells.[8,9,18–20] These transmembrane molecules characteristically consist of an extracellular ligand-binding domain connected through a segment in the plasma membrane to an intracellular tyrosine kinase domain. Binding of the ligand to the receptor results in receptor dimerization and stimulation of the receptor-associated tyrosine kinase activity, which leads to phosphorylation of tyrosine residues on both the receptor and other intracellular molecules. These changes in tyrosine phosphorylation initiate a signaling cascade leading to a variety of cellular responses. Two endothelium associated, high-affinity RTKs for VEGF have been identified, the fms-like tyrosine kinase receptor, Flt, and the kinase insert domain-containing receptor, KDR (also referred to as Flk-1 in mice).[21,22]

Blockade of VEGF signal transduction by sequestration of VEGF with antibody has been shown to prevent tumor growth.[23] VEGF RTKs must, therefore, be viewed as attractive therapeutic targets for the development

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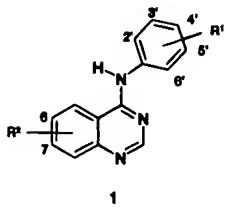


1

Figure 1.

of novel agents to treat angioproliferative diseases such as cancer.[9,10]

Recently, a series of substituted indolin-2-ones that inhibits RTKs has been reported, some of which are micromolar inhibitors of Flk-1.[24]

In this paper, we describe the synthesis and structure–activity relationships (SAR) for inhibition of tyrosine phosphorylation by anilinoquinazolines **1** (Figure 1), a novel series of extremely potent submicromolar inhibitors of VEGF receptor tyrosine kinases. Selected analogues were also evaluated in vivo in a rat uterine oedema model and for inhibition of growth of human lung carcinoma xenograft (Calu-6) in athymic mice. We also discuss a possible binding mode for these inhibitors in the catalytic domain of the VEGF RTK, based on the generated SAR and the published structural data on protein kinases.

Chemistry

The 6,7-dimethoxyanilinoquinazolines **2**–**10** (Table 1) have been prepared by multiparallel synthesis by refluxing the 4-chloroquinazoline **38**[25] with commercially available anilines in 2-propanol as described in Scheme 1.

Anilinoquinazolines possessing C-6 and C-7 substituents different from the methoxy group described in Tables 1–3 were prepared by two general strategies based on the same intermediate, namely the 7-benzyloxy-3,4-dihydroquinazolin-4-one **40**, obtained by refluxing the aminobenzamide derivative **39**[26] with Gold's reagent in dioxane (Scheme 2). In the first approach (Scheme 2, steps c–h), the anilines were coupled to the quinazoline nucleus prior to the introduction of the C-7 side chains as follows: chlorination of **40** using thionyl chloride gave the 7-benzyloxy-4-chloroquinazoline **41**. Nucleophilic displacement of the chlorine atom of **41** with anilines yielded the corresponding 7-benzyloxy-4-anilinoquinazolines **42**–**45**. Cleavage of the C-7 benzyl protecting group with either TFA or by hydrogenation using 10% Pd on charcoal led to the corresponding unprotected C-7 hydroxy-4-anilinoquinazolines **47**–**50**.

The C-7 side chains were then introduced (Scheme 2, steps g and h), either by direct alkylation of the phenol moiety with commercially available chloroalkyl or chloroaryl derivatives in DMF in the presence of potassium carbonate or by the Mitsunobu reaction using DEAD, triphenylphosphine (or *n*-butylphosphine/ADDP) in the presence of an alcohol derivative. In the case of the meta-hydroxy derivative **45**, the hydroxyl on the aniline was protected prior to the C-7 alkylation. The carbonate group was selected for this purpose due to its stability in the neutral conditions of the Mitsunobu reaction and lability under basic conditions, giving rise to **46**. After introduction of the C-7 substituent, the meta-hydroxyl function was liberated using aqueous sodium hydroxide to give **13**.

The second approach involved the introduction of the C-7 chain prior to attachment of the aniline ring (Scheme 2, steps i–n). The quinazolinone **40** was protected on N-3 using a pivaloyloxymethyl (POM) group to give **51**. Cleavage of the C-7 benzyl group was achieved by hydrogenation to give the phenol **52**. Introduction of the C-7 side chains used similar conditions to those in the first approach to give the C-7-methoxyethoxy derivative **53** and C-7-ethoxytriazole derivative **54**. Cleavage of the POM protecting group under basic conditions led respectively to the free quinazolinones **55** and **57**. Subsequent chlorination with thionyl chloride gave the corresponding C-4 chloroquinazolines **56** and **58**. Displacement of the C-4 halogen atom of the hydrochloride salt of the chloroquinazolines with anilines in protic solvent (2-propanol) led to the expected anilinoquinazolines **12** and **36** (Scheme 2).

The disubstituted derivative **14** (Scheme 3) was prepared by reacting **47** with pyridine hydrochloride to release the free catechol **59** followed by the double alkylation of the resulting C-6 and C-7 free hydroxyl using commercially available bromoethyl methyl ether in the presence of potassium carbonate in DMF (Scheme 3).

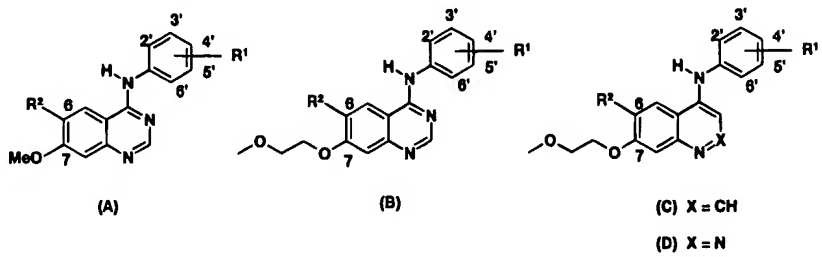
The amides **31** and **33** were obtained as described in Scheme 4. The commercially available N-protected nitro-benzamide **60** was deacylated under acidic conditions to give the anthranilic acid **61**. Reaction of **61** with formamide led to the corresponding nitroquinazolone **62**, which was then chlorinated ($SOCl_2$, DMF) to give **63**. This product was reacted with the 2-fluoro-4-chloro aniline or with the 2-fluoro-4-methyl-5-methoxycarbonyloxy aniline to give respectively **64** and **65**. Reduction of the C-7 nitro moiety followed by acylation of the resulting amine with methoxyacetyl chloride in pyridine gave **31** and **68**. The meta-hydroxy compound **33** was obtained from **68**, after hydrolysis with aqueous sodium hydroxide.

The monosubstituted C-7-methoxyethoxy derivative **15** was obtained from the fluoroquinazolinone **69**[27] by displacement of the fluorine with 2-methoxyethoxide to give **70**, which was in turn chlorinated and further reacted with the 2-fluoro-4-chloro aniline (Scheme 5).

The morpholinobutoxy derivative **23** was obtained by coupling 1-bromo-4-chlorobutane with **47** to give **72** followed by the displacement of the chlorine atom of **72** with morpholine (Scheme 6).

The 4-anilinoquinolines **16** and **17** and the 4-anilinocinnolines **18** and **19** (Scheme 7) were prepared from **78** and **83**, following a strategy similar to the first procedure used in the anilinoquinazoline series. The intermediate chloroquinoline **78** was obtained from 2-methoxy-4-nitrophenol **79** by successive alkylation with 1-bromo-2-methoxyethane followed by hydrogenation of the nitro function to give the aniline **81**. Reaction of **81** with diethylethoxymethylene malonate at 110 °C led to the intermediate aryl-enamines, which was in turn cyclized to the quinolinone **77** by a thermal decarboxylation/cyclization reaction in diphenyl ether. Finally, chlorination of **77** using standard conditions (thionyl chloride) gave the chloroquinoline **78**. A similar sequence was used to prepare the cinnolinone derivative

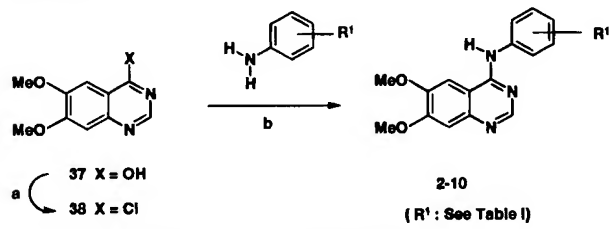
Table 1. Role of the Nucleus and Aniline Substitution on Enzyme Inhibition and Enzyme Selectivity



no.	series	R1 2′	R1 3′	R1 4′	R1 5′	R2	procedure	formula[a]	enzyme inhibition @ 2 μM ATP concentration IC_{50} μM[b] Flt	KDR	FTK	enzyme selectivity IC_{50} ratios FTK/Flt	FTK/KDR
2	A	F	H	H	H	MeO	A	$C_{16}H_{14}O_2N_3F \cdot 1.1$ HCl, 0.1 H_2O	26	2.7	>100	>4	>35
3	A	H	H	Cl	H	MeO	A	$C_{16}H_{14}O_2N_3Cl \cdot 1.1$ HCl	11	0.8	>100	>9	>120
4	A	F	H	Cl	H	MeO	A	$C_{16}H_{13}O_2N_3FCl \cdot 1.23$ HCl	2.0	0.1	7	~4	70
5	A	H	OH	H	H	MeO	A	$C_{16}H_{15}O_3N_3 \cdot 1.0$ HCl, 0.5 H_2O	0.2	0.05	11	>50	>200
6	A	F	H	I	H	MeO	A	$C_{16}H_{13}O_2N_3FI \cdot 0.88$ HCl, 0.7 H_2O	0.8				
7	A	Cl	H	I	H	MeO	A	$C_{16}H_{13}O_2N_3ClI.1.1$ HCl	>33				
8	A	F	H	F	H	MeO	A	$C_{16}H_{13}O_2N_3F_2.1.2$ HCl, 0.2 H_2O	22	2.9	58	~3	~20
9	A	H	Cl	F	H	MeO	A	$C_{16}H_{13}O_2N_3FCl.1.2$ HCl, 0.26 H_2O	>100	0.8	>100		>120
10	A	F	H	Me	OH	MeO	A	$C_{17}H_{16}N_3FO_3.1$ HCl, 0.65 iPrOH	0.03	0.003	2.5	~80	>800
11	B	F	H	Cl	H	MeO	B	$C_{18}H_{17}N_3O_3ClF \cdot 1.0$ HCl	0.4	0.007	27	>65	>3800
12	B	H	OH	H	H	MeO	A	$C_{18}H_{19}O_4N_3 \cdot 1.1$ HCl, 0.23 H_2O	0.03	<0.005	7	>200	>1400
13	B	F	H	Cl	OH	MeO		$C_{18}H_{17}N_3ClFO_4 \cdot 1.6$ H_2O	<0.002	<0.002	7.4	>3600	>3600
14	B	F	H	Cl	H	$MeO(CH_2)_2O$	B	$C_{20}H_{21}ClFN_3O_4 \cdot 1$ HCl	0.9	0.06	>100	>100	>1600
15	B	F	H	Cl	H	H	C	$C_{17}H_{15}N_3O_2FCl$	1.5	0.15	16	~10	~100
16	C	F	H	Cl	OH	MeO	D	$C_{19}H_{18}N_2O_4ClF \cdot 1.0$ HCl	0.003	<0.002	1.4	>450	>700
17	C	F	H	Cl	H	MeO	D	$C_{19}H_{18}N_2O_3ClF \cdot 0.34$ H_2O, 0.95 HCl, 0.08 iPrOH, 0.04 DMF	0.3	0.01	9.3	~30	>900
18	D	F	H	Cl	OH	MeO	D	$C_{18}H_{17}N_3O_4ClF \cdot 1.0$ HCl	0.05	0.004	>33	>650	>8000
19	D	F	H	Cl	H	MeO	D	$C_{18}H_{17}N_3O_3ClF \cdot 1.0$ HCl	63	1.1	14	~0.2	~13

[a] The C,H,N, analysis were obtained for every compounds and were within ±0.4% of the theoretical values unless otherwise stated. [b] Values are averages from at least three independent dose-response curves; variation was generally ±10% for Flt and ±20% for KDR and FTK.

Scheme 1[a]



[a] (a) $SOCl_2$/DMF/reflux; (b) iPrOH/reflux.

83. The hydroxybenzophenone **73** was alkylated with 1-bromo-2-methoxyethane under the same conditions used for the nitrophenol derivative **79**. Subsequent nitration of **79** followed by the reduction of the nitro group using iron powder in acetic acid at 100 °C yielded the 2-aminobenzophenone **76**. Diazotation followed by decomposition of the diazonium salt led to the ring closed cinnolinone **82**, which after chlorination using standard conditions (thionyl chloride) gave **83**. Displacement by the appropriate aniline using standard conditions afforded 4-anilinoquinolines **16** and **17** and the 4-anilinocinnolines **18** and **19**.

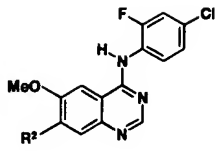
The anilines **86** and **89** were prepared as described in Scheme 8. After protection of the hydroxyl group of the 2-chloro-4-fluorophenol and 4-fluoro-2-methylphenol as methyl carbonates, **84** and **87** were nitrated using nitric acid in sulfuric acid to give the nitro derivatives **85** and **88**. In the case of derivative **85**, the phenol protecting group was cleaved by the conditions used in the work up. The nitro derivatives **85** and **88** were reduced using hydrogenation with iron powder in the presence of iron sulfate or platinum oxide respectively to give the free amines **86** and **89**.

Results and Discussion

For clarity of discussion, data on only a limited set of compounds are presented in Tables 1–4 and are used to describe the SAR. When trends are exemplified by single pairs of compounds, it is to be understood that more examples[28,29] have been obtained that support the SAR described below.

Screening of Zeneca's compound collection led to the discovery of a series of C-4 anilinoquinazolines as micromolar inhibitors of the Flt enzyme as illustrated by **2** and **3** (Table 1). Following this discovery, directed robotic synthesis confirmed the potential of this novel series and allowed rapid investigation of aniline ring substitution. From this work and subsequent optimization emerged some general trends: small lipophilic substituents such as halogens or methyl are preferred at the C-4′ position of the aniline ring (Table 1, comparison of **2** and **3**, **4** and **8**)(Table 3, comparison of **30**, **34**, and **36**), while small substituents such as fluorine are preferred at the C-2′ position (Table 1, **3**, **4**, **6**, and **7**) and hydrogen or small hydrophilic groups

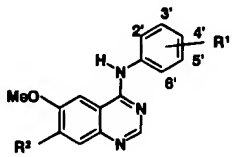
Table 2. Role of the C-7 Side Chain on Enzyme Inhibition



no.	R^2	procedure	formula[a]	enzyme inhibition @ 2μM ATP concentration IC_{50} μM[b] Flt	KDR	FTK	enzyme selectivity IC_{50} ratios FTK/Flt	FTK/KDR
11	$MeO(CH_2)_2O$	B	see Table 1	0.4	0.007	27	>65	>3800
20	1-pyrrolidinyl-$(CH_2)_2O$		$C_{21}H_{22}N_4O_2ClF$	0.3	0.04	9.4	>30	>230
21	4-morpholinyl-$(CH_2)_2O$	B	$C_{21}H_{22}N_4O_3FCl \cdot 2.0$ HCl	1.1	0.04	19	>15	>450
22	4-morpholinyl-$(CH_2)_3O$	B	$C_{22}H_{24}N_4O_3FCl \cdot 1.0$ HCl, 0.5 C_3H_6O[c]	0.2	0.009	6.7	>30	>740
23	4-morpholinyl-$(CH_2)_4O$		$C_{23}H_{26}ClFN_4O_3 \cdot 1.3\ H_2O$, 1.8 HCl	0.3	0.04	1.5	5	>35
24	4-morpholinyl-$(CH_2)_2$-O-$(CH_2)_2O$	E	$C_{23}H_{26}N_4O_4ClF \cdot 1.0\ H_2O$, 1.95 HCl	0.5	0.06	20	40	>330
25	3-thienyl-CH_2O	E	$C_{20}H_{15}N_3O_2ClFS \cdot 0.95$ HCl	0.8	0.008	>50	>60	>6250
26	4-pyridyl-CH_2O	B	$C_{21}H_{16}N_4O_2ClF \cdot 0.5$ H2O, 1.95 HCl	1.2	0.06	>33	>25	>550
27	1-imidazolyl-$(CH_2)_2O$	F	$C_{20}H_{17}N_5O_2ClF \cdot 0.4\ H_2O$, 2.0 HCl	0.1	0.04	2.6	>25	65
28	4-pyridyl-N(Me)-$(CH_2)_2O$	G	$C_{23}H_{21}N_5O_2ClF \cdot 0.9\ H_2O$, 2.0 HCl	0.1	0.006	1.6	16	>260
29	1-(1,2,4-triazolyl)-$(CH_2)_2O$	G	$C_{19}H_{16}N_6O_2ClF \cdot 1.6\ H_2O$, 1.0 HCl, 0.35 iPrOH	0.5	0.1	25	50	250
30	1-(1,2,3-triazolyl)-$(CH_2)_2O$	G	$C_{19}H_{16}ClFN_6O_2 \cdot 1.2$ HCl, 0.3 H_2O	0.3	0.01	1.8	6	180
31	$MeOCH_2CONH$		$C_{18}H_{16}ClFN_4O_3 \cdot 0.07$ HCl, 1.5 H_2O	>100	>100	>50		
32	$MeN(CH_2\text{-}CH_2)_2CH$-O	H	$C_{21}H_{22}N_4O_2Cl_3 \cdot 1.8$ HCl, 0.4 H_2O	15	1.5	40	~3	~30
33	$MeOCH_2CONH$[d]		$C_{19}H_{19}N_4O_4F \cdot 1.0$ HCl, 0.6 H_2O	0.3	0.3	>100	>330	>330

[a] The C,H,N, analysis were obtained for every compounds and were within ±0.4% of the theoretical values unless otherwise stated. [b] Values are averages from at least three independent dose-response curves; variation was generally ±10% for Flt and ±20% for KDR and FTK. [c] C: calcd, 55.1%; found, 54.7%. [d] 2′-F, 4′-Me, 5′-OH.

Table 3. Role of the Aniline Substituent in the C-7 Triazole-ethoxy Series and HUVEC Activity



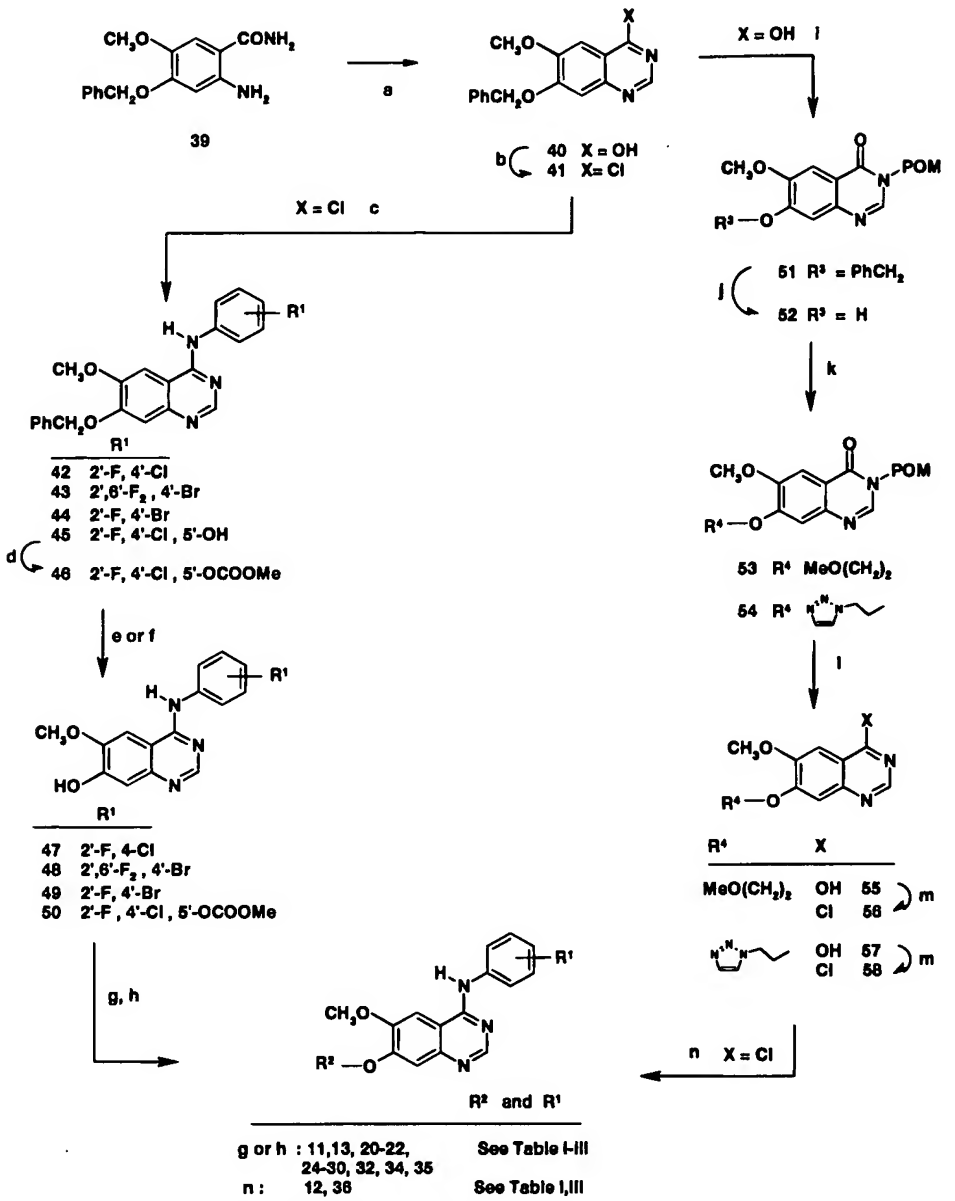
no.	R^1 2′	4′	5′	6′	R^2	procedure	formula[a]	enzyme inhibition @ 2μM ATP concentration IC_{50} μM[b] Flt	KDR	FTK	inhibition of HUVEC cell growth IC_{50} μM[c] stimulated by VEGF	FGF	unstimulated basal
13	F	Cl	OH	H	$MeO(CH_2)_2O$		see Table 1	<0.002	<0.002	7.4	0.004	0.8	5
30	F	Cl	H	H	1-(1,2,3-triazolyl)-$(CH_2)_2O$	G	see Table 2	0.3	0.01	1.8	0.04	1.7	5
34	F	Br	H	H	1-(1,2,3-triazolyl)-$(CH_2)_2O$	G	$C_{19}H_{16}BrFN_6O_2 \cdot 0.46\ H_2O$, 0.85 HCl	0.7	0.03	>100	0.05	1.5	>10
35	F	Br	H	F	1-(1,2,3-triazolyl)-$(CH_2)_2O$	E	$C_{19}H_{15}BrF_2N_6O_2 \cdot 0.4\ H_2O$, 0.9 HCl	0.8	0.04	6.1	0.02	0.1	1
36	F	CN	H	H	1-(1,2,3-triazolyl)-$(CH_2)_2O$		$C_{20}H_{16}FN_7O_2 \cdot 1.25$ HCl, 0.3 H_2O	8.1	0.3	45	0.6	3	>10

[a] The C,H,N, analysis were obtained for every compounds and were within ±0.4% of the theoretical values unless otherwise stated. [b] Values are averages from at least three independent dose-response curves; variation was generally ±10% for Flt and ±20% for KDR and FTK. [c] Values are averages from at least three independent dose-response curves; variation was generally ±15% for VEGF and FGF.

are best at the meta positions (C3′ or C5′) (Table 1, comparison of **3**–**5**, **8**, **9**, and **10**). From this screening, it also became apparent that a meta-hydroxy substituent produced increased potency (~180-fold) particularly against Flt (Table 1, comparison of **2** and **5**, **4** and **10**, and **11** and **13**). This effect suggests a possible additional interaction with the enzyme and will be discussed latter in the modeling section.

Investigation of the C-6 and C-7 positions of the quinazoline nucleus indicated that, in general, a small-electron-donating substituent at C-6 such as methoxy is well-tolerated and preferred to hydrogen or more bulky substituents as illustrated by the comparison of **11**, **14**, and **15** (Table 1). A C-6 hydrogen led to a ~20-fold reduction in KDR potency, while introduction of a bulky, flexible methoxyethoxy side chain led to a ~10-fold drop in KDR inhibition. In contrast to the C-6 position, a large range of substituents differing in their lipophilic, basic, electronic and steric nature were tolerated at C-7 of the quinazoline ring (Table 1 and 2). Replacement of the C-7 methoxy by a longer methoxyethoxy side chain led to 5- to >10-fold increases in Flt

Scheme 2[a]

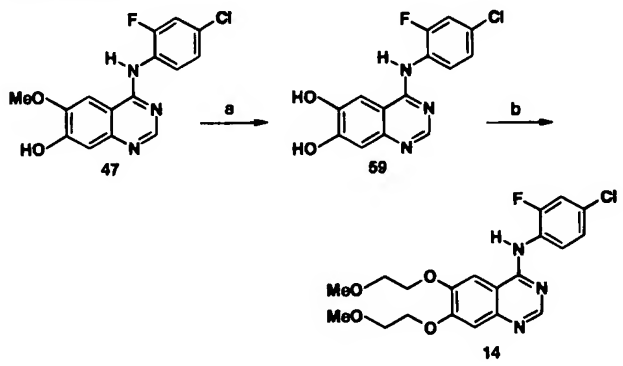


[a] (a) Gold's reagent/dioxane/reflux; (b) $SOCl_2$/DMF/reflux; (c) $ArNH_2$/iPrOH or 2-pentanol/reflux; (d) $Et_3N/(CH_3CO)_2O/CH_2Cl_2$; (e) TFA/reflux; (f) H_2/10% Pd/C; (g) R^2X/K_2CO_3/DMF/60 °C; (h) R^2OH/DEAD/Ph_3P/CH_2Cl_2 or R^2OH/ADDP/nBu_3P/CH_2Cl_2; (i) (1) NaH/DMF, (2) tBuOCOCl; (j) H_2/10% Pd/C/CH_3COOH; (k) $MeO(CH_2)_2OH$ or 1-(1,2,3-triazolyl)-$(CH_2)_2OH/Ph_3P$/DEAD/CH_2Cl_2; (l) NH_3/MeOH: (m) $SOCl_2$/DMF/reflux; (n) $ArNH_2$/iPrOH/reflux.

and KDR enzyme inhibition as indicated by the comparison of **4** and **11**, **5**, and **12** (Table 1). A similar improvement in enzyme inhibition was observed in the C-7 morpholinoalkoxy series where the propoxy side chain gave better potency than ethoxy or butoxy linkers (**21**–**24**) (Table 2). Introduction of basic and/or heteroaromatic substituents such as imidazole, triazole, or ethoxy-aminopyridine led to very potent submicromolar inhibitors of both VEGF RTK enzymes (**25**–**30**) (Table 2).[29] The ability of the C-7 position of the quinazoline to accept a large diversity of substituents suggests that this extremity of the molecule probably points toward the solvent (see modeling section).

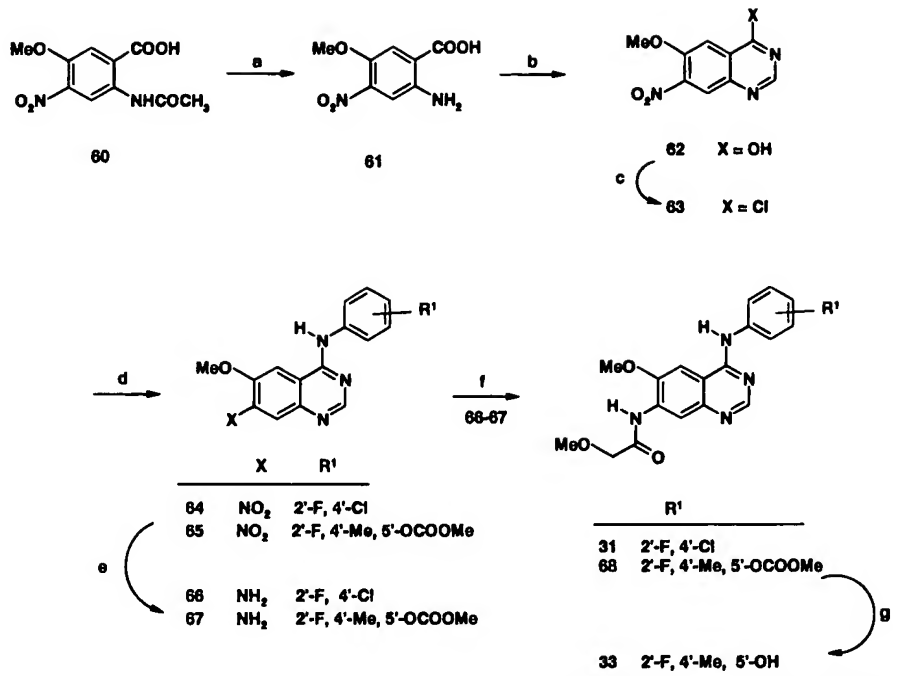
In contrast, the introduction of substituents possessing reduced flexibility at C-7 was less well tolerated in the 2-fluoro-4-chloro series and led to compounds show-

Scheme 3[a]



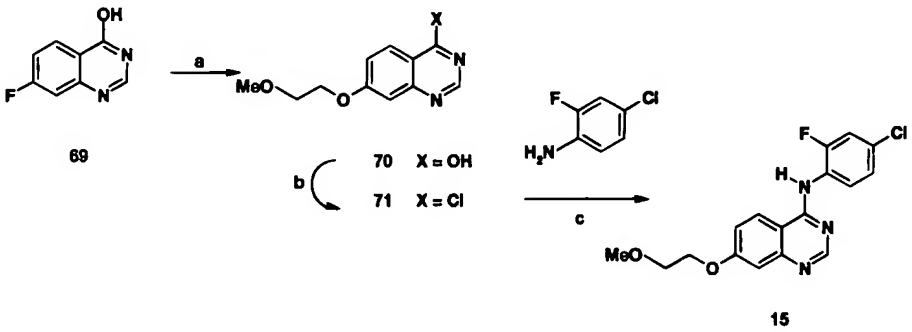
[a] (a) C_6H_5N/HCl; (b) bromoethyl methyl ether/K_2CO_3/DMF.

Scheme 4[a]



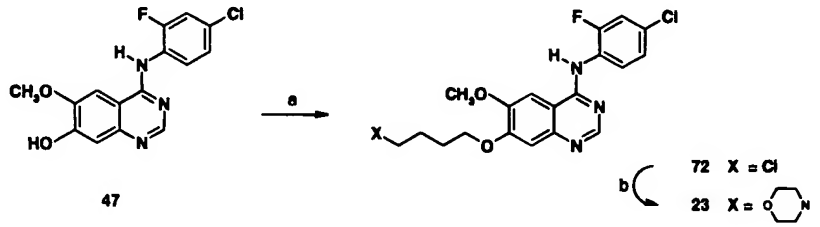
[a] (a) HCl/reflux; (b) $HCONH_2$/reflux; (c) $SOCl_2$/DMF/reflux; (d) R^1ArNH_2/iPrOH/reflux; (e) H_2/10% Pd/C/MeOH/DMF or EtOH; (f) CH_3OCH_2COCl/CH_2Cl_2/pyridine; (g) NaOH/MeOH/0 °C.

Scheme 5[a]



[a] (a) (1) $Na/CH_3O(CH_2)_2OH$, (2) reflux; (b) $SOCl_2$/DMF/reflux; (c) iPrOH/reflux.

Scheme 6[a]



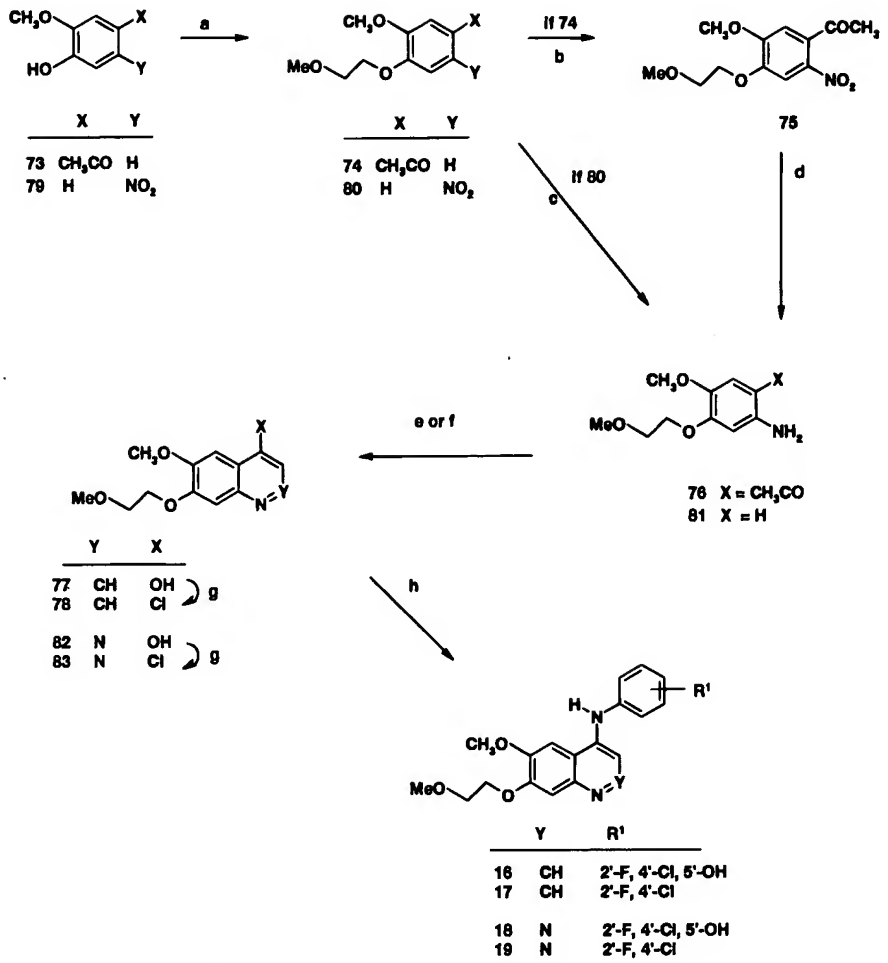
[a] (a) $Cl(CH_2)_4Br/K_2CO_3$/DMF/40 °C; (b) morpholine/110 °C.

ing reduced enzyme inhibitory properties (comparison of **11** with **31**–**32**) (Table 2). However, despite the reduced flexibility, good potency can be retained in the meta-hydroxy subseries with the C-7 amide side chains as illustrated by the comparison of **31** and **33** (Table 2), suggesting that the putative interaction of the meta-hydroxyl with the protein is strong enough to compensate in part for the C-7 constraints.

Combination of some of the best substituents on the aniline ring (e.g., 2-fluoro-4-chloro or 2-fluoro-4-methyl-5-hydroxy) and at the C-6 and C-7 positions of the quinazoline nucleus led to nanomolar inhibitors of the Flt and KDR enzymes as illustrated by **10** and **13** (Table 1).

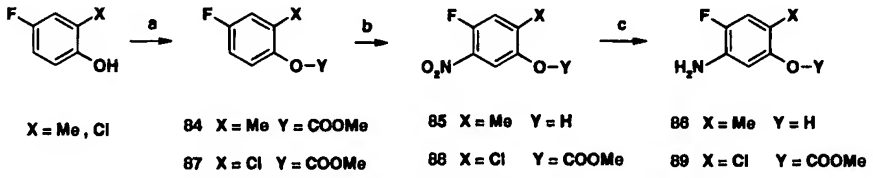
Replacement of the quinazoline nucleus was also investigated. Heteroaromatic bicycles such as quinoline or cinnoline (series C and D in Table 1) produced notable effects. In the 2-fluoro-4-chloroanilino series, the quinoline derivative is almost equipotent to the quinazoline (comparison **11** and **17**, Table 1). Replacement by a

Scheme 7[a]



[a] (a) Bromoethyl methyl ether/K_2CO_3/DMP; (b) HNO_3/2 °C; (c) H_2/10% Pd/C/EtOAc; (d) Fe/CH_3COOH/100 °C; (e) **76**: $NaNO_2/H_2SO_4$/CH_3COOH/80 °C; (f) **81**: (1) diethyl ethoxymethylenemalonate/110 °C, (2) PhOPh/240 °C; (g) $SoCl_2$/DMF/reflux; (h) $ArNH_2$/DMF/150 °C.

Scheme 8[a]



[a] (a) Methyl chloroformate/NaOH; (b) H_2SO_4/HNO_3; (c) **88**: PtO_2/EtOH, **85**: iron powder/$FeSO_4$.

cinnoline nucleus is tolerated but led to ~160-fold reduction in potency (comparison **11** and **19**, Table 1). The favorable effect of the meta-hydroxy substituent observed in the anilinoquinazoline series is also present in the quinoline series (~100-fold), leading to nanomolar inhibitors of both VEGF enzymes (comparison of **16** and **17**, Table 1). This meta-hydroxy substitution also confers submicromolar level of potency in the cinnoline series (comparison **18** and **19**, Table 1).

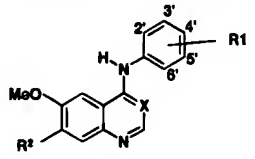
In an attempt to gain insight into the structural basis of the inhibitory activity of our series, we have developed a binding model to the enzyme that is consistent with the SAR data. This model is discussed in the modeling section below.

Enzyme and Cell Selectivity. Selectivity was measured against FGFR-1 (FTK) another important RTK thought to be important in angiogenesis.

As shown by **5**, **10**, and **11**–**14**, compounds from both the 2-fluoro-4-chloro and 3-hydroxy anilinoquinazoline series are very selective inhibitors of the VEGF RTKs, Flt, and KDR, compared to the FGF RTK with ratios of IC_{50}s ranging respectively from 50- to >3800-fold (Table 1). C-7 side chain modifications were also useful to improve the selectivity (comparison of **4** and **11**, **21**, **22**, **25**, **26**) (Table 1 and 2). Similarly, the quinoline and cinnoline series provided very selective VEGF RTK inhibitors as illustrated by KDR/FTK potency ratios ranging from ~30 to >8000-fold (**16**–**18**, Table 1).

Interestingly, although the anilinoquinazoline series had previously delivered potent EGF RTK inhibitors,[30–33] we found that distinct SAR existed for EGF RTK and VEGF RTK inhibitions. On the aniline ring, the EGF preferred substitution, namely, a lipophile (Cl, Br, ...)[25,30–33] at the C-3′ position is not optimal for VEGF

Table 4. Mouse Plasma Levels Following Oral Administration of 100 mg/kg of Compound



		R1				plasma level μM[a]	
no.	X	2'	4'	5'	R2	@ 6 h	@ 24 h
4	N	F	Cl	H	MeO	NT[b]	NT[b]
10	N	F	Cl	OH	MeO	<0.2	<0.2
11	N	F	Cl	H	$MeO(CH_2)_2O$	12	<0.1
12	N	H	H	OH	$MeO(CH_2)_2O$	NT[b]	NT[b]
13	N	F	Cl	OH	$MeO(CH_2)_2O$	<0.1	<0.1
17	CH	F	Cl	H	$MeO(CH_2)_2O$	3	NT[b]
22	N	F	Cl	H	4-morpholinyl-$(CH_2)_3O$	4	0.8
24	N	F	Cl	H	4-morpholinyl-$(CH_2)_2$-O-$(CH_2)_2O$	11	<0.1
27	N	F	Cl	H	1-imidazolyl-$(CH_2)_2O$	11	<0.1
30	N	F	Cl	H	1-(1,2,3-triazolyl)-$(CH_2)_2O$	49	10
34	N	F	Br	H	1-(1,2,3-triazolyl)-$(CH_2)_2O$	45	13

[a] Variation was generally ±15%. [b] NT: Not tested.

inhibition (**4**, **8**, and **9**, Table 1). In addition, it is noteworthy that compound **9**, a well-known extremely potent inhibitor of EGF RTK (IC_{50}: 9 nM)[33] is a 100–10000 times less active as an inhibitor of Flt and KDR (Table 1). Furthermore, **9** is >50-fold less active against Flt than its isomer **4**, which possesses one of the preferred substitutions (2-fluoro-4-chloro) for inhibition of VEGF RTKs. Similarly, the EGF preferred quinazoline substitution, namely, a large C-6 substituent[25,28,30,31,34] is not optimal for potent inhibition of the Flt and KDR enzymes (**11** and **14**).

Molecular Modeling: Development of a Binding Model for the Anilino-quinazoline, -quinoline, and -cinnoline Series. Our study relies on the key structure–activity relationships described in this paper and on the published structural data on the protein kinase family (see Experimental Section).

Recently, an increasing number of 3D structures of protein kinases, especially kinases bound to diverse competitive inhibitors, have been published, improving our knowledge of the key hydrogen bond interactions with the enzyme backbone and of the role of the conserved residues clustered near the active site.[35–47] The adenine moiety of ATP is anchored in the active site by two hydrogen bonds, one donating and one accepting. As illustrated in Figure 2, one involves the N-6 hydrogen (donating) and the backbone carbonyl of Glu-121, the other involves the N-1 of adenine (accepting) and the backbone amide of Val-123. Most of the experimental or modeled enzyme–inhibitor complexes show a similar hydrogen bond pattern.[36,39,44,48–51] In other complexes,[36,43,45] the accepting hydrogen bond is conserved, whereas the donating hydrogen bond is now closer to the entry of the active site and shared with the backbone carbonyl of the residue equivalent to Val-123 (Figure 2). In a few other cases,[40,46,47] the inhibitors are anchored in the adenine site only by the highly conserved, accepting hydrogen bond interaction. Although the H-bonding is essential for inhibitor binding, the number of protein–ligand hydrogen bonds is not correlated to potency. Modeling[48,50–52] and experimental[45–47] studies have also revealed the presence of a deep, medium size, pocket adjacent to the adenine binding

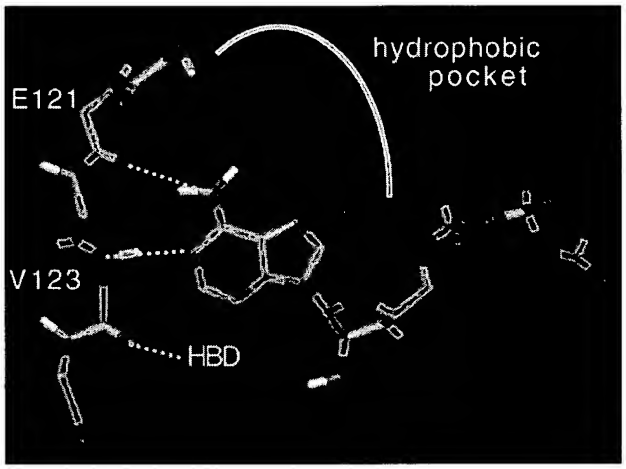


Figure 2. Schematic representation of ATP bound to the PKA active site.[53] The adenine moiety is anchored by one donating and one accepting H-bond (blue dotted lines). Other kinase-inhibitor complexes[36,43,45] have shown that another H-bond could be formed between an H-bond donor (HBD) and the backbone carbonyl of residue equivalent to Val-123 (green dotted line). The location of the hydrophobic pocket is also shown.

site. This hydrophobic pocket, made up of conserved and nonconserved residues, is not utilized by the ATP itself, but can be exploited by inhibitors from various chemical classes. It is now evident that potency and partial selectivity can be achieved by small molecule inhibitors that occupy this pocket.

The full enzyme kinetic profile of our series of VEGF RTK inhibitors has not been measured. However, results from experiments varying ATP concentration in the kinase assays are consistent with ATP competition.[28] Furthermore, it has been shown that the potent EGF–RTK inhibitor *N*-(3-chlorophenyl)-4-quinazolinylamine is competitive with respect to ATP,[30] strongly suggesting to us that our compounds inhibit the catalytic activity of Flt and KDR through a similar process. Therefore, using a 3D homology model of Flt1 based on the X-ray structure of PKA,[53] we have searched for a binding mode that would fit our compounds into the ATP binding site and be consistent with the SAR data.

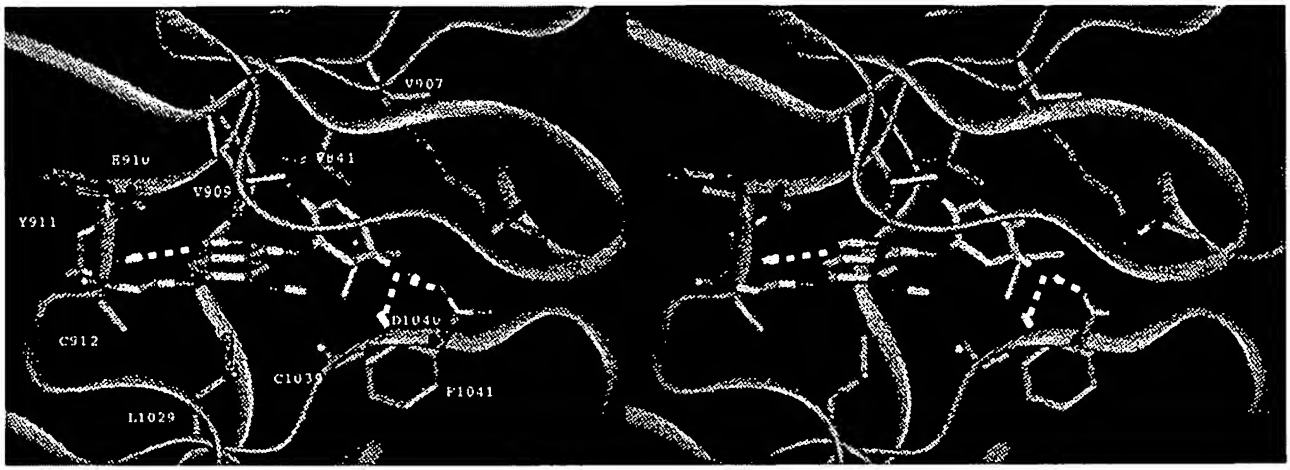


Figure 3. Stereoview of compound **13** docked into the ATP-binding site of the homology model of Flt-1. Hydrogen bond interactions are represented as dotted lines. To remain consistent with other published models, residues are numbered as in the complete sequence of Flt-1. The quinazoline ring occupies the adenine binding site, and the anilino moiety is buried into the deep hydrophobic pocket. The C-6 and C-7 side chains are oriented toward the entrance of the binding site and extend out of the pocket. The N-1 of the quinazoline nucleus forms an H-bond with the backbone NH of Cys-912 (residue homologue to Val-123 in PKA). A second H-bond may be formed between the hydroxyl group of the phenol moiety and the carboxylic function of Asp-1040 (residue equivalent to Phe-185 in PKA). In that position, the phenolic oxygen could also interact with the backbone NH of Asp-1040. Some of the residues that make hydrophobic contacts with the inhibitor are also shown.

The binding mode that was most satisfactory is illustrated in Figure 3 for the quinazoline derivative **13**. In this model, the quinazoline ring occupies the flat and rather narrow adenine site. The highly conserved accepting hydrogen bond is realized by N-1 of the quinazoline, which binds to the backbone amide of Cys-912 (equivalent to Val-123 in PKA), whereas N-3 does not participate in any hydrogen bonding. The C-2 of the quinazoline is located close to the carbonyl oxygen of Glu-910. This binding mode possibly explains the relatively lower potency observed in the cinnoline series, where C-2 is replaced by nitrogen (**11**, **19**) (Table 1), leading to unfavorable repulsive electrostatic interactions. It also possibly explains the equipotency observed between the quinazoline and the quinoline series (**11**, **17**) (Table 1), as the quinazoline N-3, which is in van der Waals contacts with Val-909, can be replaced by a carbon atom in the quinoline.

In our model (see Experimental Section), the 2-fluoro-4-chloro anilino substituent is buried in the hydrophobic pocket adjacent to the adenine site. The limited size of this pocket explains the rather limited substitution pattern allowed on the phenyl ring. The C-6 and C-7 side chains are then oriented completely opposite, toward the outside of the cleft. This is consistent with the rather broad substitution pattern on C-7, but not the more restricted substitution observed for the C-6 position. This underlines the limits of the homology model; prediction is less accurable when moving away from the highly conserved binding site.

The ~180-fold increase in potency observed in the meta-hydroxy anilinoquinazoline, quinoline, and cinnoline derivatives (**12**, **13**, **16**, **18**) (Table 1) suggests the existence of an extra interaction with the enzyme. In the binding mode we propose, this hydroxy group forms a hydrogen bond with the flexible side chain of Asp-1040, a residue of the highly conserved segment -Asp-Phe-Gly, which normally participates in MgATP coordination. In this position, the phenolic oxygen could also interact with the backbone NH of Asp-1040. The submicromolar level of potency in the otherwise moderately active cinnoline series suggests that this interaction slightly reorients the molecule in the active site so that the repulsive electrostatic interactions with Cys-912 does not occur or is significantly reduced. The beneficial effect of an hydroxy in an analogous position has also been observed in a series of pyrazolopyrimidine, inhibitors of EGF RTK.[50] In their model, the authors assume that the phenol moiety occupies the deep hydrophobic pocket and suggest the existence of an hydrogen bond between the hydroxyl group and the backbone of Phe-832 (in their EGF RTK model, equivalent to Phe1041 in our Flt1 model). An analogous hydrogen bond has also been experimentally identified in the complex of FGFR1 with PD173074,[45] a pyrido[2,3-*d*]pyrimidine derivative; while the 6-(3,5-dimethoxy)-phenyl substituent is buried in the hydrophobic pocket, one of the methoxy oxygens interacts with the amide nitrogen of Asp-641 (Asp-1040 in Flt1). Interestingly, minimal or more pronounced shifts in the backbone and side chains of these conserved Asp and Phe has been observed upon inhibitor binding.[35,38,39,41] All these observations suggested that depending on the chemical class of inhibitor, the molecular model used and on the kinase in question, different hydrogen bonds can be formed with this Asp-Phe segment. However, while it is reasonable to assume that this segment can provide strong hydrogen bond interactions with inhibitors it remains difficult to define its exact location, as the molecular modeling technique is fairly limited in predicting conformational changes or more subtle adjustment of specific residue positions upon inhibitor binding.

Cellular Activity. Our most potent and selective kinase inhibitors were evaluated for their ability to inhibit the incorporation of tritiated thymidine during the growth of human umbilical vein endothelial cells (HUVECs) stimulated by VEGF in vitro. As shown in Table 3, the potency observed against the one or more isolated enzymes translated to a submicromolar level of inhibition of stimulated cell growth. Moreover, an

Table 5. Inhibition of Rat Uterus Weight Gain Following Administration of VTK Inhibitors

no.	dose (mg/kg) @ −18 and −1 h	% inhibition of uterus weight gain	significance (p)
4	100	34	<0.05
10	60	80	<0.05
11	100	31	<0.05
12	100	NA[a]	
22	100	59	<0.05
34	NT[b]		

[a] NA: not active. [b] NT: not tested.

excellent level of selectivity is conserved in cells as indicated by the 5- to ~200-fold selectivity ratios observed between the inhibition of VEGF and FGF stimulated HUVEC growth (**13**, **30**, **34**) (Table 3). The slight reduction in selectivity observed between enzyme and cell data may be explained by differences in ATP concentrations between the enzyme assay and whole cells. Interestingly, inhibition of KDR RTK alone appeared to be sufficient to provide inhibition of VEGF signaling in HUVECs (**34**, **35**, **36**) (Table 3). The 50- to 1250-fold higher concentrations needed to inhibit the growth of unstimulated HUVECs (basal IC_{50}) compared to those required for the inhibition of VEGF stimulated HUVECs growth indicates that these compounds do not impart any direct cytostatic or cytotoxic effect (Table 3).

Plasma Levels in Mice. As shown in Table 4, the 2-fluoro-4-chloro-anilinoquinazolines listed are bioavailable orally when dosed to mice at 100 mg/kg and achieved good total plasma levels as illustrated by the concentrations obtained at 6 h. Comparison of plasma levels of quinazoline derivative **11** and quinoline **17** indicates a clear difference in this species between the two series, the latter showing lower plasma levels in mice, which may be a consequence of a higher degree of metabolism.[28] In contrast to the 2-fluoro-4-chloroanilino series, in mice, the meta-hydroxy anilinoquinazoline series plasma levels (**13**) were below the limit of detection at 6 h. Evaluation of the plasma levels following oral dosing in rat of key representatives of the meta-hydroxy series showed a similar pattern. In some cases, the glucuronide of the phenol moiety was detected suggesting a very high degree of conjugation of this meta-hydroxy subseries.

Heteroaromatic C-7 side chains such as imidazole and triazole led to high-plasma levels as shown respectively by **27** and **30**, **34**. In these latter cases, total plasma levels are maintained above 10 μM for as long as 24 h (Table 4).

Activity in Vivo. In immature rats, the initial increase in uterine weight observed following administration of estradiol is primarily due to tissue oedema induced by expression of VEGF in the uterus. A VEGF sequestering agent (VEGF m-Ab, 100 μg/rat) administered intraperitoneally 18 h prior to estradiol treatment inhibit this early uterotrophic effect by up to ~80%. The test used to evaluate our inhibitors measured their capacity to reduce the acute increase in uterine weight at 5 h following oestrogen stimulation.

As shown in Table 5, when administered orally at a dose of 100 mg/kg at 18 h and 1 h prior to estradiol injection, the 2-fluoro-4-chloro anilinoquinazoline series significantly inhibited the increase in weight of the rat uterus. Up to 59% inhibition was obtained with **22**. The most profound effect was observed with the most potent kinase inhibitor from the meta-hydroxy series **10**, which exhibited ~80% inhibition with two doses of 60 mg/kg. This was surprising given its plasma pharmacokinetic profile in mice (Table 5) and suggested that the pharmacokinetics of such compounds may be different in rat. In this latter species, following oral dosing (100 mg/kg), **10** showed low but detectable 6 h plasma levels (~1 μM), which in this accute model and due to the excellent intrinsic potency of this molecule is probably sufficient to explain the level of efficacy observed. Because of the PK differences observed between rat and mouse as well as the accute effect seen in this test, the rat uterine oedema assay was used as a measure of VEGF pharmacology but appeared not as discriminatory to predict efficacy in chronic models such as xenografts.

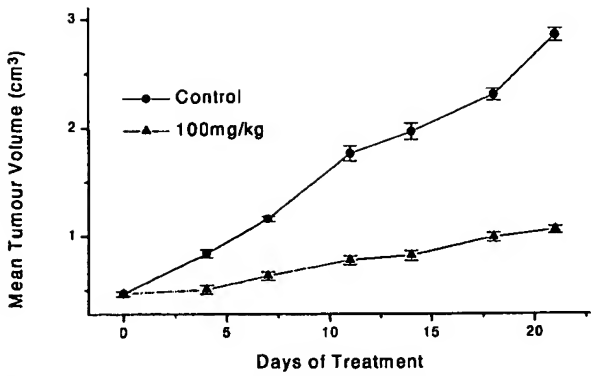


Figure 4. Growth inhibition of established Calu-6 tumor xenografts treated with **34**. Nude mice bearing Calu-6 xenografts received a daily oral dose of vehicle (●) or 100 mg/kg **34** (▲). Data points represent the mean (±SEM) of 10 (control) and 9 (treated) mice respectively.

VEGF RTK inhibitors were also evaluated for their ability to inhibit growth of human tumors implanted subcutaneously in athymic mice. Oral administration of **34** (100 mg/kg/day) for 21 days to mice with established Calu-6 lung carcinoma (0.5 cm^3) xenografts markedly inhibited tumor growth by 75% ($p < 0.001$, one tailed t-test) (Figure 4), without causing any loss of animal condition. It is noteworthy that **34** does not inhibit the in vitro growth of Calu-6 cells ($IC_{50} > 70\ \mu M$) at levels of compounds likely to be freely available in vivo (Mouse SPB: 98% ± 0.5%)(mean ± SE, $n = 5$), which indicates that even taking into account a likely accumulation of the compound, the tumor growth inhibition observed cannot be attributed to a direct cytotoxic or cytostatic effect on tumor cells.

Comparable efficacy has since been confirmed on other human tumor xenografts including colon, lung, breast, prostate, and ovary tumors.[54]

Conclusions

Novel anilinoquinazolines carrying small lipophilic 2′ and 4′ substituents, a C-6 methoxy, and a wide range of C-7 substituents are nanomolar inhibitors of the VEGF receptor tyrosine kinase enzymes. Addition of a meta-hydroxy group on the aniline nucleus enhances the potency of this series of molecules.

Using a molecular model of VEGF RTK, Flt-1, a possible binding mode for this series of inhibitors has

been identified that is consistent with the observed SAR and published structural data on kinase-inhibitor complexes.

Most of these derivatives are potent submicromolar inhibitors of human endothelial cell proliferation stimulated by VEGF. The anilinoquinazolines and quinolines are highly selective inhibitors of Flt and KDR tyrosine kinase (up to >1000-fold), in comparison to FGF RTK and this enzyme selectivity profile translates well into cell selectivity.

Many of the anilinoquinazolines are orally absorbed in rats and mice and are effective in inhibiting the acute, VEGF-mediated, uterotrophic response to estradiol in rats, and the growth of human xenograft tumors in athymic mice. The anilinoquinolines are less effective in vivo probably because of rapid metabolism.

The anilinoquinazoline **34** (ZD4190) was identified as one of the most promising representatives of this new series of molecules. In view of its biological properties and efficacy in vivo, this compound was selected for development and is currently undergoing preclinical studies to further define its efficacy and toxicology profile.

Experimental Section

Protein Modeling. The first 3D crystal structure of the catalytic domain of a protein kinase to become available was that of the cAMP-dependent protein kinase A, PKA.[53] The crystal structure revealed a bilobal shape, with a large cleft separating the two lobes and providing the binding site for ATP. The adenine ring is buried in the hydrophobic bottom of the cleft, while the sugar and triphosphate moieties extend toward the opening of the cleft. This typical architecture was evident in subsequent crystal structures,[55-58] indicating a conserved ATP binding mode, in agreement with the high degree of sequence similarity displayed by the kinase catalytic core.[59] However, the structure of the ternary complex PKA–ATP–PKI[52] was, for a while, the only kinase structure available in its active closed form;[60] therefore, it has been extensively used as the reference for homology modeling of other members of the protein kinase family. These models have been successfully utilized to understand SAR data and guide medicinal chemistry programs.[48-52,61]

The 3D model of human VEGF RTK, Flt-1, was built with the automated homology modeling program Modeler[62] interfaced with the molecular modeling software Quanta.[63] The crystal structure of the ternary complex of PKA (PDB entry 1ATP) was used as a template.[53] The basis for the 3D model was the sequence alignment with the kinase family; all the key and conserved residues have been aligned and most of the deletions or insertions have been assigned using the published multiple sequence alignment of kinases.[59] However, Flt-1 has two large insertions, one near the beginning of the C-terminal domain (Asp926–Glu992), the other in the C-terminal tail (Phe1182–Leu1232). As it is reasonable to think that they are not part of the active site and as they are too large to be modeled, we have removed them from the original sequence.

In the PKA structure, the nonconserved carboxy-terminal tail extends over the surface from the large lobe to the top of the small lobe. In the closed ternary complex, residue Phe327 of the C-terminal TSNFDDY motif makes part of the adenosine binding pocket. Experimental data have shown that Phe327 is expelled from its position upon binding of the inhibitor staurosporine.[41] In the open binary complex PKA-PKI, the C-terminal segment, and Phe327 in particular, undergoes an even larger shift, leaving the adenosine site more accessible to the solvent.[60] Intriguingly, Flt-1 has a similar TSMFDDY sequence in its carboxy-terminal tail, whereas KDR itself, which has 70% sequence similarity over the kinase domain with Flt-1, does not have this motif. Moreover, none of the other known human PTKs have this motif. Therefore, it is not clear whether this similarity is fortuitous or not and we do not know if this segment occupies a similar location to that observed in the 3D structure of PKA. In Flt-1, the presence of a large insertion preceding this segment could modify its position. The C-terminal sequence has been, however, included in our original homology model of Flt-1. When docking our inhibitors, it is obvious that steric clashes occur between the C7-side chains and the Phe1039 (equivalent to Phe327 in PKA). This suggested to us that either the 3D location of this segment is wrong or this segment is subject to large conformational changes as experimentally observed in PKA.[41,60] Therefore, we have truncated the model for our subsequent docking studies. Before that, a final check and a geometry optimization of the amino acids chains lying within the active site was performed, using the CHARMm force field[64,65] implemented in Quanta 97.[63] The VEGF RTK inhibitors were built in Quanta and the charges were assigned by the Quanta charge template method.[63] These inhibitors have been docked manually into the ATP binding site, and the amino acid side chains were reoriented when unfavorable steric interactions occurred. The most relevant solutions were then energy minimized with the CHARMm force field to relieve remaining unfavorable steric contacts.

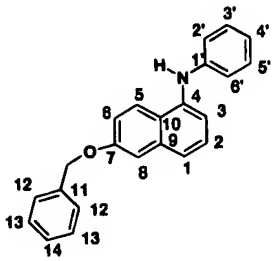


Figure 5.

Very recently, the structure of the kinase domain of human KDR has been published[66] (PDB entry 1VR2). The authors have compared the ATP binding site of KDR and FGFR1 and came to the conclusion that the overall architecture of the site is conserved. As the coordinates of KDR are not yet available, we have checked our binding mode by docking our compounds into the ATP binding site of the FGFR1 structure (PDB entry 1FGK).[36] All the key interactions we have described previously are conserved, which justify the use of PKA as a template for our homology model of Flt-1.

General Procedures. All experiments were carried out under an inert atmosphere and at room temperature unless otherwise stated. Flash chromatography was carried out on Merck Kieselgel 60 (Art. 9385). The purities of compounds for test were assessed by analytical HPLC on a Hichrom S5ODS1 Sperisorb Column System set to run isocratically with 60–70% MeOH + 0.2% CF_3COOH in H_2O as eluent. TLCs were performed on precoated silica gel plates (Merck Art. 5715), and the resulting chromatograms were visualized under UV light at 254 nm. Melting points were determined on a Kofler Block or with a Büchi melting point apparatus on compounds isolated as described in the experimental procedures and are uncorrected. The NMR spectra were determined in Me_2SO-d_6 solution (unless otherwise stated) on a Bruker AM 200 (200 MHz) spectrometer or on a JEOL JNM EX 400 (400 MHz). For the ^{13}C NMR spectra, ring carbon atoms have been numbered as shown below. For ^1H NMR spectra, hydrogens have been given the numbering of the carbon atom they are attached to (Figure 5).

Chemical shifts are expressed in unit of δ (ppm), and peak multiplicities are expressed as follows: s, singlet; d, doublet; dd, doublet of doublet; t, triplet; br s, broad singlet; m, multiplet. Fast atom bombardment (FAB) mass spectra were determined with a VG MS9 spectrometer and Finnigan Incos data system, using Me_2SO as the solvent and glycerol as the matrix or with a Finnigan SSQ 7000 for the electro-spray technique. With the appropriate mode, either positive or

negative ion data could be collected. NMR and mass spectra were run on isolated intermediates and final products and are consistent with the proposed structures. For the microanalysis, all the adducts mentionned were measured: water was measured by the Karl-Fisher method using a Mettler DL 18; HCl content was determined by titration using silver nitrate solution and a Metrohm 686 and the organic adducts were measured by ^{1}H NMR.

The anilines used (2-fluoro-4-chloro; 4-chloro; 2-fluoro; 4-chloro; 3-hydroxy; 2-chloro-4-iodo; 2,4-difluoro; 3-chloro-4-fluoro; 2,4-difluoro; 3-chloro-4-fluoro; 2-fluoro-4-bromo; 2,6-difluoro-4-bromo) were commercially available. 4-(2-Chloroethyl)morpholine hydrochloride, 1-(2-chloroethyl)pyrrolidine hydrochloride, 3-thiophenemethanol and 4-(chloromethyl)-pyridine hydrochloride were purchased from Aldrich. The 4-(3-chloropropyl)morpholine was prepared as described in ref 67.

The following abreviations have been used: DMF: *N,N*-dimethylformamide; DEAD: diethylazodicarboxylate; ADDP: 1,1′-(azodicarbonyl)dipiperidine; Gold's reagent: [3-(dimethylamino)-2-azaprop-2-en-1-ylidene]dimethylammonium chloride; TFA: trifluoroacetic acid; DMSO: dimethyl sulfoxide.

The anilinoquinazolines **2**–**10** were prepared by multiparallel synthesis. Eight reactions were conducted in parallel in 10 mL tubes. The tubes were heated in a 10 holes heater. The experimental details of this reaction are described below for the preparation of compound **2**.

***N*-(2-Fluorophenyl)-6,7-dimethoxy-4-quinazolinylamine 2. (Procedure A).** To a mixture of 2-fluoroaniline (136 mg, 1.22 mmol) in 2-propanol (6 mL) was added 5.5 N hydrogen chloride in 2-propanol (0.22 mL) followed by 4-chloro-6,7-dimethoxyquinazoline **38**[25] (247 mg, 1.1 mmol). After heating at 80 °C for 4 h, the mixture was cooled and ether (5 mL) was added. The solid was filtered, washed with ether (2 × 5 mL), and dried under vacuum overnight at 50 °C to give 338 mg of **2** (91%). ^{1}H NMR: δ 4.05 (s, 6H, 2 CH_3O), 7.3–7.5 (m, 3H, H3′, H4′ and H5′), 7.4 (s, 1H, H8), 7.6 (t, 1H, H6′), 8.3 (s, 1H, H5), 8.8 (s, 1H, H2). ^{13}C NMR: δ 56.5 (O–CH_3), 56.9 (O–CH_3), 99.6 (C8), 104.0 (C5), 106.9 (C10), 116.3 (d, C3′, J C–F = 19.6 Hz), 124.4 (d, C1′, J C–F = 7.7 Hz), 124.8 (C5′ or C4′), 128.8 (C4′ or C5′), 129.2 (d, C6′, J C–F = 7.7 Hz), 135.4 (C4), 148.7 (C2), 150.3 (C9), 156.5 (C7), 156.9 (d, C2′, J C–F = 249.1 Hz), 159.1 (C6). MS–ESI *m/z* 300 $[MH]^+$. Anal. ($C_{16}H_{14}O_2N_3F$·1.1 HCl, 0.1 H_2O) C, H, N.

A similar procedure was used to prepare **3**–**10**, **12**, **64**, and **65**. In the case of **64** and **65**, the corresponding chloroquinazoline hydrochloride was used and the reaction was carried out without addition of hydrogen chloride in 2-propanol.

***N*-(4-Chloro-2-fluorophenyl)-6-methoxy-7-(2-methoxyethoxy)-4-quinazolinylamine hydrochloride 11. (Procedure B).** 2-Bromoethyl-methyl ether (712 μL, 7.56 mmol) was added dropwise to a solution of *N*-(4-chloro-2-fluorophenyl)-7-hydroxy-6-methoxy-4-quinazolinylamine **47** (2.2 g, 6.88 mmol) and potassium carbonate (2.84 g, 20.6 mmol) in DMF (110 mL). The mixture was stirred for 10 h at 60 °C, then for 2 days at ambient temperature. The solvent was removed by evaporation, and the crude product purified by flash chromatography eluting with ethyl acetate/petroleum ether (4:1). The resulting solid was dissolved in hot ethanol and 3 N hydrogen chloride in ethanol was added. After cooling, the resulting solid was collected by filtration, washed with ethanol, and dried under vacuum to give 1.74 g of **11** (62%). Mp 255–257 °C. ^{1}H NMR (DMSO-d_6; CD_3COOD): δ 3.36 (s, 3H, CH_3O), 3.79 (t, 2H, CH_2O), 4.02 (s, 3H, CH_3O), 4.34 (t, 2H, CH_2O), 7.33 (s, 1H, H8), 7.46 (dd, 1H, H3′), 7.60–7.68 (m, 2H, H6′ and H5′), 8.15 (s, 1H, H5), 8.79 (s, 1H, H2). ^{13}C NMR: δ 57.1 (Ph–O–CH_3), 58.5 (CH_2–O–CH_3), 68.8 (Ph–O–CH_2), 69.9 (CH_3–O–CH_2), 100.5 (C8), 104.4 (C5), 107.2 (C10), 117.0 (d, C3′, J C–F = 23.6 Hz), 123.8 (d, C1′, J C–F = 12.2 Hz), 125.1 (d, C5′, J C–F = 3.08 Hz), 130.1 (C6′), 132.6 (d, C4′, J C–F = 9.4 Hz), 135.9 (C4), 149.0 (C2), 150.6 (C9), 159.9 (C7), 158.5 (d, C2′, J C–F= 250.5 Hz), 159.2 (C6). MS–ESI *m/z* 378–380 $[MH]^+$. Anal. ($C_{18}H_{17}N_3O_3ClF$·1.0 HCl) C, H, N.

A similar procedure was used to prepare **14**, **21**, **22**, **26**, **72**, and **73**. The heating conditions used were respectively overnight at 30 °C; 100 °C for 3 h; 110 °C for 4 h; 60 °C for 2 h; 40 °C for 5 h; 50 °C overnight. In the case of **26**, KI (0.28 mmol) was added to facilitate the reaction. In the case of **22**, **72**, and **73**, the crude product was purified by column chromatography prior to hydrochloride salt formation.

***N*-(4-Chloro-2-fluoro-5-hydroxyphenyl)-6-methoxy-7-(2-methoxyethoxy)-4-quinazolinylamine 13.** 1,1′-Azodicarbonyl-dipiperidine (413 mg, 1.6 mmol) was added portionwise to a stirred mixture of *N*-(5-acetoxy-4-chloro-2-fluorophenyl)-7-hydroxy-6-methoxy-4-quinazolinylamine **50** (250 mg, 0.66 mmol), 2-methoxyethanol (63 mL, 0.8 mmol), and tributylphosphine (405 mL, 1.6 mmol) in methylene chloride at 0 °C. The resulting solution was allowed to warm to ambient temperature and stirred for 2 h. The solid was removed by filtration, the solvent was removed from the filtrate by evaporation and the residue purified by flash chromatography eluting with acetonitrile/methylene chloride (1:9, increasing in polarity to 4:6) to give 180 mg of *N*-(5-acetoxy-4-chloro-2-fluorophenyl)-7-(2-methoxyethoxy)-6-methoxy-4-quinazolinylamine (62%) as a solid.

A solution of concentrated aqueous ammonia (5 mL) was added to a solution of *N*-(5-acetoxy-4-chloro-2-fluorophenyl)-7-(2-methoxyethoxy)-6-methoxy-4-quinazolinylamine (180 mg, 0.4 mmol) in methanol (50 mL). The mixture was stirred at ambient temperature for 3 h and then diluted with water. Most of the methanol was removed by evaporation, and the resulting precipitate collected by filtration, washed with water, and dried to give 73 mg of **13** (45%). Mp >250 °C. ^{1}H NMR: δ 3.29 (s, 3H, CH_3O), 3.74 (t, 2H, CH_2O), 3.94 (s, 3H, CH_3O), 4.28 (t, 2H, CH_2O), 7.15 (d, 1H, H6′), 7.19 (s, 1H, H5), 7.38 (d, 1H, H3′), 7.77 (s, 1H, H8), 8.36 (s, 1H, H2), 9.40 (s, 1H, OH). MS–ESI *m/z* 394 $[MH]^+$. Anal. ($C_{18}H_{17}N_3ClFO_4$·1.6 H_2O) C, H, N.

***N*-(4-Chloro-2-fluorophenyl)-7-(2-methoxyethoxy)-4-quinazolinylamine 15. (Procedure C).** A solution of 4-chloro-7-(2-methoxyethoxy)quinazoline hydrochloride **71** (624 mg, 2.27 mmol) and 4-chloro-2-fluoroaniline (305 μL, 2.6 mmol) in 2-propanol (20 mL) was heated at reflux for 30 min. The solvent was removed by evaporation, and the residue partitioned between ethyl acetate and water. The organic layer was separated, washed with aqueous sodium bicarbonate solution, water, dried ($MgSO_4$), and the solvent removed by evaporation. The residue was triturated with ether to give 662 mg of **15** (84%) as a white solid. Mp 140–141 °C. ^{1}H NMR: δ 3.35 (s, 3H, CH_3O), 3.74 (t, 2H, CH_2O), 4.29 (t, 2H, CH_2O), 7.21 (s, 1H, H8), 7.28 (d, 1H, H6), 7.35 (d, 1H, H3′), 7.6 (m, 2H, H5′ and H6′), 8.36 (d, 1H, H5), 8.43 (s, 1H, H2), 9.75 (s, 1H, NH). ^{13}C NMR: δ 58.1 (CH_2–O–CH_3), 67.4 (Ph–O–CH_2), 70.1 (CH_3–O–CH_2), 107.4 (C8), 109.0 (C10), 116.6 (d, C3′, J C–F = 24.2 Hz), 118.1 (C6), 124.5 (d, C5′, J C–F = 31 Hz), 124.6 (C5), 125.7 (d, C1′, J C–F = 11.5 Hz), 129.3 (C6′), 130.2 (d, C4′, J C–F = 10.3 Hz), 152.0 (C9), 155.0 (C2), 156.6 (d, C2′, J C–F = 25.1 Hz), 158.0 (C4), 162.0 (C7). MS–ESI *m/z* 347 $[MH]^+$. Anal. ($C_{17}H_{15}N_3O_2FCl$) C, H, N.

***N*-(4-Chloro-2-fluoro-5-hydroxyphenyl)-6-methoxy-7-(2-methoxyethoxy)-4-quinolinylamine hydrochloride 16. (Procedure D).** A suspension of 4-chloro-2-fluoro-5-hydroxyaniline (270 mg, 1.64 mmol) and 4-chloro-6-methoxy-7-(2-methoxyethoxy)quinoline hydrochloride **78** (500 mg, 1.64 mmol) in DMF (6 mL) was heated at 150 °C for 5 h, and the solvent was removed by evaporation. The residue was triturated with ether and collected by filtration. The solid was washed with water, ether and dried under vacuum to give 475 mg of **16** (67%). ^{1}H NMR: δ 3.37 (s, 3H, CH_3O), 3.8 (t, 2H, CH_2O), 4.03 (s, 3H, CH_3O), 4.31 (t, 2H, CH_2O), 6.5 (m, 1H, H3), 7.2 (d, 1H, H3′), 7.49 (s, 1H, H8), 7.64 (d, 1H, H6′), 8.16 (s, 1H, H5), 8.40 (d, 1H, H2), 10.66 (s, 1H, OH), 10.82(s, 1H, NH).^{13}C NMR: δ 56.6 (Ph–O–CH_3), 58.2 (CH_2–O–CH_3), 68.3 (Ph–O–CH_2), 69.8 (CH_2–O–CH_3), 99.9 (C3), 100.4 (C8), 102.7 (C5), 111.5 (C10), 114.9 (C6′), 118.0 (d, C3′, J C–F = 23.4 Hz), 118.9 (d, C4′, J C–F = 9.4 Hz), 123.8 (d, C1′, J C–F = 14.2 Hz), 135.1 (C4), 139.9 (C2), 149.3 (d, C2′, J C–F = 237.2 Hz), 149.6 (C6), 150.6 (d, C5′, J C–F = 3.5 Hz), 153.1 (C9), 153.9 (C7). MS–ESI *m/z* 393 $[MH]^+$. Anal. ($C_{19}H_{18}N_2O_4ClF$·1.0 HCl) C, H, N.

A similar procedure was used to prepare **17**–**19**. Reaction mixture were heated respectively for 30, 45, and 20 min.

***N*-(4-Chloro-2-fluorophenyl)-6-methoxy-7-[2-(pyrrolidin-1-yl)ethoxy]-4-quinazolinylamine 20.** 1-(2-Chloroethyl)pyrrolidine hydrochloride (200 mg, 1.2 mmol) was added to a mixture of *N*-(4-chloro-2-fluorophenyl)-7-hydroxy-6-methoxy-4-quinazolinylamine **47** (403 mg, 1.26 mmol) and potassium carbonate (650 mg, 4.7 mmol) in DMF (4 mL). The mixture was heated to 100 °C and further portions of 1-(2-chloroethyl)-pyrrolidine hydrochloride (800 mg in total) were added periodically over 4 h, while the reaction mixture was maintained at 100 °C. The reaction was then allowed to cool and volatile components were removed by evaporation. The residue was partitioned between methylene chloride and water, separated, and the organic phase passed through phase separating paper. Column chromatography eluting with methylene chloride/methanol (95:5) gave 50 mg of **20** (10%). ^{1}H NMR: δ 1.8–2.1 (m, 4H, 2-CH_2), 3.1 (m, 2H, CH_2N), 3.55–3.7 (m, 4H, 2-CH_2N), 4.05 (s, 3H, CH_3O), 4.6 (t, 2H, CH_2O), 7.4 (m, 2H, H3′ and H8), 7.58 (d, 1H, H5′), 7.65 (dt, 1H, H6′), 8.5 (s, 1H, H5), 8.8 (s, 1H, H2).^{13}C NMR: δ 22.6 (2C, pyrrolidine CH_2), 52.1 (N–CH_2–CH_2–O), 53.9 (2C, pyrrolidine N–CH_2), 57.3 (Ph–O–CH_3), 64.9 (Ph–O–CH_2), 101.0 (C8), 104.68 (C5), 107.6 (C10), 116.9 (d, C3′, J C–F = 24.2 Hz), 123.8 (d, C1′, J C–F = 12.6 Hz), 125.0 (d, C5′, J C–F = 3.2 Hz), 129.8 (C6′), 132.3 (d, C4′, J C–F = 10.4 Hz), 135.8 (bs, C4), 149.0 (C2), 150.2 (C9), 154.6 (C7), 156.8 (d, C2′, J C–F = 251.9 Hz), 164.8 (C6). MS–ESI m/z 417 $[MH]^+$. Anal. ($C_{21}H_{22}N_4O_2ClF$) C, H, N.

***N*-(4-Chloro-2-fluorophenyl)-6-methoxy-7-[4-(4-morpholinyl)-butoxy]-4-quinazolinylamine hydrochloride 23.** A solution of *N*-(4-chloro-2-fluorophenyl)-7-(4-chlorobutoxy)-6-methoxy-4-quinazolinylamine **72** (0.1 g, 0.24 mmol) in morpholine (2 mL) was heated at 110 °C for 2 h. After cooling, the mixture was partitioned between ethyl acetate and water. The organic layer was washed with water, brine, dried ($MgSO_4$), filtered, and evaporated. The resulting oil was purified by column chromatography eluting with methylene chloride/methanol 92:8. The resulting solid was dissolved in methylene chloride and 2 N hydrogen chloride in ether was added (1 mL). The solution was concentrated to a third of its volume and the solid was filtered, washed with ether, and dried under vacuum to give 87 mg of **23** (68%). ^{1}H NMR (DMSO-d_6, CF_3COOD): δ 1.95 (bs, 4H, CH_2CH_2), 3.1 (t, 2H, CH_2O morpholine), 3.25 (bs, 2H, CH_2O morpholine), 3.5 (d, 2H, CH_2O Ar), 3.75 (t, 2H, CH_2N), 3.95 (d, 2H, CH_2N morpholine), 4.03 (s, 3H, CH_3O), 4.28 (t, 2H, CH_2N morpholine), 7.41 (s, 1H, H8), 7.45 (d, 1H, H3′), 7.62 (t, 1H, H6′), 7.68 (dd, 1H, H5′), 8.2 (s, 1H, H5), 8.88 (s, 1H, H2).^{13}C NMR: δ 19.7 (CH_2–CH_2–O), 25.4 (N–CH_2–CH_2), 50.9 (2C, CH_2–N–CH_2), 55.5 (N–CH_2–CH_2), 57.1 (Ph–O–CH_3), 63.1 (2C, CH_2–O–CH_2), 68.6 (Ph–O–CH_2), 100.4 (C8), 104.3 (C5), 107.1 (C10), 116.9 (d, C3′, J C–F = 24.0 Hz), 123.8 (d, C1′, J C–F = 11.30 Hz), 125.0 (d, C5′, J C–F = 4.3 Hz), 129.9 (C6′), 132.3 (d, C4′, J C–F = 8.9 Hz), 135.9 (C4), 148.7 (C2), 150.3 (C9), 155.7 (C7), 156.8 (d, C2′, J C–F = 253.5 Hz), 158.9 (C6). MS–ESI m/z 461 $[MH]^+$. Anal. ($C_{23}H_{26}ClFN_4O_3$·1.3 H_2O, 1.8 HCl) C, H, N.

***N*-(4-Chloro-2-fluorophenyl)-6-methoxy-7-[2-[2-(4-morpholinyl)-ethoxy]ethoxy]-4-quinazolinylamine hydrochloride 24. (Procedure E).** Diethyl azodicarboxylate (209 mg, 1.2 mmol) was added dropwise to a mixture of *N*-(4-chloro-2-fluorophenyl)-7-hydroxy-6-methoxy-4-quinazolinylamine **47** (128 mg, 0.4 mmol), triphenylphosphine (314 mg, 1.2 mmol), and 2-(2-morpholinoethoxy)ethanol[68] (97 mg, 0.56 mmol) in methylene chloride (4 mL) under nitrogen. The mixture was stirred for 1 h at ambient temperature, triphenylphosphine (105 mg, 0.4 mmol), 2-(2-morpholinoethoxy)ethanol (49 mg, 0.28 mmol), and diethyl azodicarboxylate (70 mg, 0.4 mmol) were added. The mixture was stirred for 1 h at ambient temperature and was purified by pouring directly onto a silica column eluting with methylene chloride/acetonitrile/methanol (6:3:1). The purified product was triturated with ether, collected by filtration, and dissolved in methylene chloride. 2 N hydrogen chloride in ether (0.5 mL) was added, and the resulting precipitate was collected by filtration, washed with ether, and dried under vacuum to give 100 mg of **24** (45%). ^{1}H NMR (DMSO-d_6, CF_3COOD): δ 3.1–3.2 (m, 2H, CH_2N), 3.3–3.5 (m, 5H, 2-CH_2N and CHO), 3.7–3.8 (m, 2H, CH_2O), 3.9–4.0 (m, 5H, CH_2O), 4.02 (s, 3H, CH_3O), 4.4 (br s, 2H, CH_2O), 7.46 (s, 1H, H8), 7.48 (d, 1H, H3′), 7.6 (t, 1H, H6′), 7.7 (d, 1H, H5′), 8.25 (s, 1H, H5), 8.89 (s, 1H, H2). MS–ESI m/z 477 $[MH]^+$. Anal. ($C_{23}H_{26}N_4O_4ClF$·1.0 H_2O, 1.95 HCl) C, H, N.

A similar procedure was used to synthesize **25** and **35**.

***N*-(4-Chloro-2-fluorophenyl)-7-[2-(1-imidazolyl)-ethoxy]-6-methoxy-4-quinazolinylamine hydrochloride 27. (Procedure F).** A solution of 1,1′-(azodicarbonyl)dipiperidine (378 mg, 1.5 mmol) in methylene chloride (5 mL) was added dropwise to a suspension of *N*-(4-chloro-2-fluorophenyl)-7-hydroxy-6-methoxy-4-quinazolinylamine **47** (160 mg, 0.5 mmol), tributylphosphine (303 mg, 1.5 mmol), and 2-(imidazol-1-yl)-ethanol (67 mg, 0.6 mmol)[69] in methylene chloride (8 mL), and the mixture was stirred for 3 h at ambient temperature. Acetic acid (60 mg, 1 mmol) was added, and the solvent was removed by evaporation. The solid residue was adsorbed on silica and purified by column chromatography eluting with methylene chloride/methanol (9:1 followed by 8:2). The resulting white solid was dissolved in methylene chloride/methanol and a solution of 5 N hydrogen chloride in 2-propanol was added. The solvent was removed by evaporation and the solid was triturated with ether, filtered, washed with ether, and dried under vacuum to give **27** (180 mg, 74%). mp 218–221 °C. ^{1}H NMR: δ 4.01 (s, 3H, CH_3O), 4.62 (t, 2H, CH_2O), 4.76 (t, 2H, CH_2N), 7.44 (dd, 1H, H3′), 7.48 (s, 1H, H8), 7.59 (t, 1H, H6′), 7.66 (dd, 1H, H5′), 7.72 (s, 1H, imidazole H4), 7.84 (s, 1H, imidazole H5), 8.41 (s, 1H, H5), 8.78 (s, 1H, H2), 9.22 (s, 1H, imidazole H2). MS–ESI m/z 414 $[MH]^+$. Anal. ($C_{20}H_{17}N_5O_2$-ClF·0.4 H_2O, 2,0 HCl) C, H, N.

***N*-(4-Chloro-2-fluorophenyl)-6-methoxy-7-[2-[1-(1,2,4-triazolyl)]-ethoxy]-4-quinazolinylamine hydrochloride 29. (Procedure G).** Diethylazodicarboxylate (295 μL, 1.8 mmol) was added dropwise to a solution of *N*-(4-chloro-2-fluorophenyl)-7-hydroxy-6-methoxy-4-quinazolinylamine **47** (300 mg, 0.93 mmol), 2-(1,2,4-triazol-1-yl)ethanol[70] (159 mg, 1.4 mmol), and triphenylphosphine (492 mg, 1.8 mmol) in methylene chloride (10 mL). The mixture was stirred for 2 h at ambient temperature and further triphenylphosphine (246 mg, 0.9 mmol) and diethylazodicarboxylate (147 μL, 0.9 mmol) were added. The mixture was stirred for 1 h at ambient temperature, and the resulting precipitate was collected by filtration, washed with methylene chloride and ether, and dried under vacuum. This solid was suspended in methylene chloride/methanol and a 5 M solution of hydrogen chloride in 2-propanol (1.0 mL) was added. The volatiles were removed by evaporation, and the residue was triturated with ether. The resulting solid was collected by filtration, washed with ether, and dried under vacuum to give 219 mg of **29** (52%). Mp 169–174 °C. ^{1}H NMR: δ 3.99 (s, 3H, CH_3O), 4.60 (t, 2H, CH_2N), 4.74 (t, 2H, CH_2O), 7.43 (d, 1H, H3′), 7.45 (s, 1H, H8), 7.59 (t, 1H, H6′), 7.67 (dd, 1H, H5′), 8.06 (s, 1H, triazole), 8.41 (s, 1H, H5), 8.68 (s, 1H, triazole), 8.83 (s, 1H, H2). MS–ESI m/z 415 $[MH]^+$. Anal. ($C_{19}H_{16}N_6O_2ClF$·1.6 H_2O, 1.0 HCl, 0.35 ^{i}PrOH) C, H, N.

***N*-(4-Bromo-2-fluorophenyl)-6-methoxy-7-[2-(1*H*-1,2,3-triazol-1-yl)ethoxy]-4-quinazolinylamine hydrochloride 34.** To a solution of *N*-(4-bromo-2-fluorophenyl)-7-hydroxy-6-methoxy-4-quinazolinylamine **49** (0.13 g, 0.357 mmol) in methylene chloride (4 mL) was added triphenylphosphine (0.28 g, 1.07 mmol) and 2-hydroxyethyl-1-(1,2,3-triazole)[71] (60 mg, 0.53 mmol) followed by diethylazodicarboxylate (0.17 mg, 1.08 mmol) dropwise. After the solution was stirred for 1 h at ambient temperature, triphenylphosphine (95 mg, 0.36 mmol), 2-hydroxyethyl-1-(1,2,3-triazole) (20 mg, 0.177 mmol) and diethylazodicarboxylate (60 μL, 0.381 mmol) was added. After the solution was stirred for 1 h at ambient temperature, the precipitate formed was filtered. The solid was suspended in a mixture of methylene chloride/methanol 1:1 and 3.8 N hydrogen chloride in ether (0.5 mL) was added. The solution was diluted with ether. The precipitate was filtered, washed with ether, and dried under vacuum at 70 °C to give 96 mg of **34**

(54%). ^{1}H NMR spectrum (DMSO-d_6, CF_3COOD): δ 3.98 (s, 3H, CH_3O), 4.69 (t, 2H, CH_2O), 4.95 (t, 2H, CH_2N), 7.35 (s, 1H, H8), 7.5–7.65 (m, 2H, H6′ and H3′), 7.78 (s, 1H, triazole), 7.82 (d, 1H, H5′), 8.07 (s, 1H, triazole), 8.21 (s, 1H, H5), 8.87 (s, 1H, H2). ^{13}C NMR: δ 48.7 (N–CH_2), 56.4 (Ph–O–CH_3), 67.1 (Ph–O–CH_2), 102.5 (C5), 108.4 (C8), 109.2 (C10), 117.8 (d, C4′, J C–F = 9.1 Hz), 119.5 (d, C3′, J C–F = 23.4 Hz), 125.5 (triazole C5), 126.5 (d, C1′, J C–F = 12.3 Hz), 127.7 (d, C6′, J C–F = 2.9 Hz), 129.7 (d, C5′, J C–F = 2.1 Hz), 133.6 (triazole C4), 146.9 (C9), 149.1 (C6), 153.0 (C7), 153.2 (C2), 156.8 (d, C2′, J C–F = 251.5 Hz), 157.1 (C4). MS–ESI m/z 459–461 $[MH]^+$. Anal. ($C_{19}H_{16}BrFN_6O_2 \cdot 0.46\ H_2O$, 0.85 HCl) C, H, N.

N-[4-(4-Chloro-2-fluoroanilino)-6-methoxy-7-quinazolinyl]-2-methoxyacetamide hydrochloride 31. To a suspension of N-(2-fluoro-4-chlorophenyl)-7-amino-6-methoxy-4-quinazolinylamine **66** (0.4 g, 1.1 mmol) and pyridine (5 mL) in methylene chloride (8 mL) cooled at 5 °C was added methoxyacetyl chloride (123 μL, 1.3 mmol). After the solution was stirred for 2 h at ambient temperature, the volatile components were removed by evaporation. The residue was triturated with water and filtered. The solid was azeotroped successively with ethanol and toluene. The crude product was purified by column chromatography, eluting with methylene chloride/acetonitrile/methanol 60:38:2. After evaporation of the solvent, the solid was dissolved in methylene chloride and 3 N hydrogen chloride in 2-propanol was added. The volatiles were removed by evaporation, and the solid was filtered and washed with 2-propanol, followed by ether and dried under vacuum to give 119 mg of **31** (40%). ^{1}H NMR: δ 3.45 (s, 2H, CH_2), 4.12 (s, 3H, CH_3O), 4.19 (s, 3H, CH_3O), 7.45 (d, 1H, H3′), 7.6 (t, 1H, H6′), 7.7 (d, 1H, H5′), 8.4 (s, 1H, H8), 8.8 (s, 1H, H5), 8.9 (s, 1H, H2), 9.52 (s, 1H, NH), 11.52 (bs, 1H, NH).^{13}C NMR: δ 57.8 (Ph–O–CH_3), 85.8 (CH_2–O–CH_3), 71.4 (C=OCH_2–O), 104.2 (C8), 106.9 (C5), 108.8 (C10), 116.9 (d, C3, J C–F = 23.6 Hz), 123.7 (d, C1′, J C–F = 12.4 Hz), 126.0 (d, C5′, J C–F = 4.2 Hz), 129.8 (C6), 132.4 (d, C4′, J C–F = 9.7 Hz), 134.9 (C4), 135.2 (C7), 148.7 (C2), 149.3 (C9), 156.8 (d, C2′, J C–F = 252.1 Hz), 159.2 (C6), 169.2 (C=O). MS–ESI m/z 391 $[MH]^+$. Anal. ($C_{18}H_{16}ClFN_4O_3 \cdot 0.7$ HCl 1.5 H_2O) C, H, N.

A similar procedure was used to synthesize **28** and **30**.

N-(4-Chloro-2-fluorophenyl)-6-methoxy-7-[(1-methyl-4-piperidinyl)oxy]-4-quinazolinylamine 32. (Procedure H). To a suspension of N-(4-chloro-2-fluorophenyl)-7-hydroxy-6-methoxy-4-quinazolinylamine **47** (159 mg, 0.5 mmol) in methylene chloride (5 mL) cooled at 5 °C was added triphenylphosphine (328 mg, 1.25 mmol), and N-methyl-4-hydroxypiperidine (115 mg, 1 mmol), followed by diethylazodicarboxylate (218 mg, 1.25 mmol) dropwise. After the solution stirred for 1 h at ambient temperature, the volatiles were removed by evaporation and the residues were partitioned between ether and 2 N aqueous hydrochloric acid. The aqueous layer was washed with ether and the pH was adjusted to 9 with aqueous sodium bicarbonate. The aqueous layer was extracted with methylene chloride. The organic layer was washed with brine, dried ($MgSO_4$), and evaporated. The residue was purified by column chromatography on neutral alumina eluting with methylene chloride/methanol 97:3. After evaporation of the solvent, the solid was dissolved in methylene chloride and 3 N hydrogen chloride in 2-propanol was added. The volatiles were removed by evaporation and the solid was filtered, washed with 2-propanol, followed by ether and dried under vacuum to give 180 mg of **32** (79%). ^{1}H NMR (DMSO-d_6, CD_3COOD): δ 1.9–2.0 (m, 1H), 2.15–2.25 (m, 2H), 2.35–2.45 (m, 1H), 2.82 and 2.85 (2s, 3H, CH_3N), 3.05–3.25 (m, 2H), 3.4–3.5 (d, 1H), 3.55–3.65 (d, 1H), 4.0 and 4.05 (2s, 3H, CH_3O), 4.85 (m, 0.5H), 5.0 (bs, 0.5H), 7.45 (d, 1H, H3′), 7.5 (s, 0.5H, H8), 7.58 (s, 0.5H, H8), 7.6 (t, 1H, H6′), 7.68 (dd, 1H, H5′), 8.18 (s, 0.5H, H5), 8.2 (s, 0.5H, H5), 8.9 (s, 1H, H2). MS–ESI m/z 417 $[MH]^+$. Anal. ($C_{21}H_{22}N_4O_2Cl_3 \cdot 1.8$ HCl, 0.4 H_2O) C, H, N.

N-(2-Fluoro-5-hydroxy-4-methylphenyl)-6-methoxy-7-methoxyacetamido-4-quinazolinylamine hydrochloride 33. 2 N aqueous sodium hydroxide solution (620 μL) was added dropwise to a suspension of N-(2-fluoro-5-methoxycarbonyloxy-4-methylphenyl)-6-methoxy-7-methoxyacetamido-4-quinazolinylamine **68** (275 mg, 0.62 mmol) in methanol (8 mL) at 5 °C, and the mixture then stirred for 90 min at ambient temperature. The reaction mixture was diluted with water and adjusted to pH 7 with 2 N hydrogen chloride. The precipitated solid was collected by filtration, resuspended in ethanol and a 5 N solution of hydrogen chloride in 2-propanol (0.3 mL) added. The volatiles were removed from the resulting solution by evaporation, and the solid washed with ether collected by filtration and dried under vacuum to give 216 mg of **33** (82%). Mp 300–306 °C. ^{1}H NMR: δ 2.18 (s, 3H, CH_3–Ar), 3.47 (s, 3H, CH_3O), 4.13 (s, 3H, CH_3O), 4.21 (s, 2H, CH_2O), 6.92 (d, 1H, H6′ or H3′), 7.13 (d, 1H, H3′or H6′), 8.41 (s, 1H, H8), 8.80 (s, 1H, H5), 8.90 (s, 1H, H2), 9.54 (s, 1H, NH or OH), 9.72 (s, 1H, NH or OH), 11.49 (s, 1H, NH).^{13}C NMR: δ 15.7 (Ph–O–CH_3), 57.6 (CO–CH_2–O), 71.4 (C=OCH_2–O), 104.0 (C8), 106.8 (C5), 108.6 (C10), 113.4 (C6′), 117.3 (d, C3′, JC–F = 20.6 Hz), 121.2 (d, C1′, J C–F = 14.2 Hz), 125.1 (d, C4′, J C–F = 7.7 Hz), 134.6 (C4), 135.0 (C7), 148.6 (C2), 149.3 (C9), 149.7 (d, C2′, J C–F = 238.4 Hz), 151.5 (d, C5′, J C–F = 1.7 Hz), 159.2 (C6), 169.2 (C=O). MS–ESI m/z 387 $[MH]^+$. Anal. ($C_{19}H_{19}N_4O_4F \cdot 1.0$ HCl, 0.6 H_2O) C, H, N.

N-(4-Cyano-2-fluorophenyl)-6-methoxy-7-[2-(1H-1,2,3-triazol-1-yl)ethoxy]-4-quinazolinylamine hydrochloride 36. A solution of 4-chloro-6-methoxy-7-[2-(1,2,3-triazol-1-yl)-ethoxy]quinazoline **58** (170 mg, 0.56 mmol) and 4-cyano-2-fluoroaniline[72] (91 mg, 0.67 mmol) in 2-propanol (8 mL) containing 3 N hydrogen chloride in 2-propanol (0.2 mL) was refluxed for 2.5 h. After the solution was cooled to ambient temperature, methylene chloride (20 mL) and methanol (20 mL) were added. A21 amberlyste resin was added until pH = 8. The mixture was filtered. Silica was added to the filtrate and the solvents were removed under vacuum. The residue was poured onto a silica column and eluted successively with methylene chloride; methylene chloride/ethyl acetate 1:1 and methylene chloride/ethyl acetate/methanol 6:4:1. After removal of the solvent, the residue was dissolved in methylene chloride/methanol (5:1) and 3 N hydrogen chloride in ether (0.5 mL) was added. The volatiles were removed by evaporation and the residue was filtered, washed with ether, and dried under vacuum to give 72 mg of **36** (28%). ^{1}H NMR (DMSO-d_6, CF_3COOD): 4.01 (s, 3H, CH_3O), 4.71 (t, 2H, CH_2O), 4.98 (t, 2H, CH_2N), 7.40 (s, 1H, H5), 7.8 (s, 1H, triazole), 7.8–7.9 (m, 2H, H3′ and H6′), 8.1 (d, 1H, H5′), 8.15 (s, 1H, triazole), 8.25 (s, 1H, H8), 8.93 (s, 1H, H2).^{13}C NMR: δ 48.5 (CH_2–N), 57.3 (Ph–O–CH_3), 67.8 (Ph–O–CH_2), 101.1 (C8), 104.7 (C5), 107.9 (C10), 110.8 (d, C4′, J C–F = 10.8 Hz), 117.7 (d, CN, J C–F = 2.3 Hz), 120.6 (d, C3′, J C–F = 23.4 Hz), 125.6 (triazole C5), 129.5 (d, C5′ or C6′, J C–F = 3.5 Hz), 129.6 (C6′ or 5′), 130.1 (d, C1′, J C–F = 11.4 Hz), 136.3 (C4), 149.1 (C2), 150.6 (C9), 155.3 (C7), 156.3 (d, C2′, J C–F = 252.2 Hz), 158.9 (C6). MS–ESI m/z 406 $[MH]^+$. Anal. ($C_{20}H_{16}FN_7O_2 \cdot 1.25$ HCl, 0.3 H_2O) C, H, N.

4-Chloro-6,7-dimethoxyquinazoline hydrochloride 38[25]. (Procedure I). A solution of 6,7-dimethoxy-3,4-dihydroquinazolin-4-one **37** (2.06 g, 10 mmol) in thionyl chloride (20 mL) containing DMF (2 drops) was stirred and heated to reflux for 2 h. The mixture was evaporated and the residue was triturated with ether, filtered, and dried under vacuum to give 2 g of **38** (90%). ^{1}H NMR: δ 4.0 (s, 3H, CH_3O), 4.01 (s, 3H, CH_3O), 7.40 (s, 1H, H8 or H5), 7.46 (s, 1H, H8 or H5), 8.88 (s, 1H, H2). MS–EI m/z 224–226 $[M]^+$.

A similar procedure was used to prepare **40**, **56**, **58**, **63**, **71**, and **78**. In the case of **56** and **58**, the free base of the chloroquinazoline was generated by treatment with sodium bicarbonate.

7-Benzyloxy-6-methoxy-3,4-dihydroquinazolin-4-one 40. A mixture of 2-amino-4-benzyloxy-5-methoxybenzamide **39**[26] (10 g, 40 mmol) and Gold's reagent (7.4 g, 50 mmol) in dioxane (100 mL) was stirred and heated at reflux for 24 h. Sodium acetate (3.02 g, 37 mmol) and acetic acid (1.65 mL, 29 mmol) were added to the reaction mixture and it was heated for a further 3 h. The volatile components were removed by evapo-

ration, water was added to the residue, the solid was collected by filtration, washed with water, and dried. Recrystallization from acetic acid gave 8.7 g of **40** (84%). Mp 266 °C. ^{1}H NMR: δ 3.85 (s, 3H, CH_3O), 5.25 (s, 2H, CH_2O), 7.25 (s, 1H, H8), 7.4 (m, 6H, Ph and H5), 7.95 (s, 1H, H2), 12.0 (br s, 1H, NH). ^{13}C NMR: δ 55.7 (CH_2O), 70.0 (OCH_3), 105.1 (C5), 109.3 (C8), 115.7 (C10), 127.9 (2 C12), 128.0 (C14), 128.5 (2 C13), 136.3 (C11), 143.8 (C2), 144.7 (C6), 148.7 (C7), 153.3 (C9), 160.0 (C4). MS−ESI *m/z* 305 $[MNa]^+$. Anal. ($C_{16}H_{14}N_2O_3 \cdot 0.24\ H_2O$) C, H, N.

***N*-7-Benzyloxy-(4-chloro-2-fluorophenyl)-6-methoxy-4-quinazolinylamine hydrochloride 42. (Procedure J).** A solution of 7-Benzyloxy-4-chloro-6-methoxyquinazoline hydrochloride **41** (1.2 g, 3.5 mmol) and 4-chloro-2-fluoroaniline (444 μL, 4 mmol) in 2-propanol (40 mL) was refluxed for 1.5 h. After the solution was cooled, the precipitate was collected by filtration, washed with 2-propanol then ether and dried under vacuum to give 1.13 g of **42** (71%). Mp 239−242 °C. ^{1}H NMR: δ 4.0 (s, 3H, CH_3O), 5.36 (s, 2H, CH_2O), 7.39−7.52 (m, 9H, Ph and H3′, H5′, H6′and H8), 8.1 (s, 1H, H5), 8.75 (s, 1H, H2). ^{13}C NMR: δ 57.0 (O−CH_3), 70.7 (O−CH_2), 100.8 (C8), 104.3 (C5), 107.1 (C10), 116.9 (d, C3′, J C−F = 23.8 Hz), 123.8 (d, C1′, J C−F = 13.4 Hz), 125.0 (d, C5′, J C−F = 3.0 Hz), 128.3 (C13), 128.4 (C14), 128.6 (C12), 129.9 (C6′), 132.4 (d, C4′, J C−F = 9.1 Hz), 135.2 (C11), 135.5 (C4), 148.8 (C2), 150.5 (C9), 155.4 (C7), 156.8 (d, C2′, J C−F = 252.2 Hz), 159.0 (C6). MS−ESI *m/z* 410 $[MH]^+$. Anal. ($C_{22}H_{17}N_3O_2ClF \cdot 1.0$ HCl) C, H, N.

A similar procedure was used to prepare **43**−**45**. In the case of **44** and **45**, the mixture was heated for 4 h and 2 h, respectively.

***N*-(5-Acetoxy-4-chloro-2-fluorophenyl)-7-benzyloxy-6-methoxy-4-quinazolinylamine 46.** Triethylamine (216 mL, 1.5 mmol) and acetic anhydride (133 mL, 1.4 mmol) were added to a stirred suspension of *N*-7-benzyloxy-(4-chloro-2-fluoro-5-hydroxyphenyl)-6-methoxy-4-quinazolinylamine hydrochloride **45** (600 mg, 1.4 mmol) in methylene chloride (7 mL). The mixture was stirred at ambient temperature for 3 h and insoluble material removed by filtration. Volatiles were removed from the filtrate by evaporation, and the residue purified by flash chromatography, eluting with methylene chloride/methanol (100:0, increasing in polarity to 97:3) to give 340 mg of **46** (52%). ^{1}H NMR: δ 2.34 (s, 3H, CH_3CO), 3.94 (s, 3H, CH_3O), 5.28 (s, 2H, CH_2O), 7.28 (s, 1H, H8), 7.35−7.44 (m, 2H, H3′ and H14), 7.50 (d, 2H, H12), 7.58 (d, 1H, H6′), 7.70 (d, 1H, H13), 7.80 (s, 1H, H5), 8.37 (s, 1H, H5′), 9.30 (s,-1H, H2). MS−ESI *m/z* 468 $[MH]^+$.

***N*-(4-Chloro-2-fluorophenyl)-7-hydroxy-6-methoxy-4-quinazolinylamine 47. (Procedure K).** A solution of *N*-(4-chloro-2-fluorophenyl)-7-benzyloxy-6-methoxy-4-quinazolinylamine hydrochloride **42** (892 mg, 2 mmol) in TFA (10 mL) was refluxed for 50 min. After the solution was cooled, the mixture was poured onto ice. The precipitate was collected by filtration, dissolved in methanol (10 mL) and basified to pH = 11 with aqueous ammonia. After concentration by evaporation, the solid product was collected by filtration, washed with water then ether and dried under vacuum to give 460 mg of **47** (72%). Mp 141−143 °C. ^{1}H NMR: δ 3.95 (s, 3H, CH_3O), 7.05 (s, 1H, H8), 7.35 (d, 1H, H3′), 7.54−7.59 (m, 2H, H5′ and H6′), 7.78 (s, 1H, H5), 8.29 (s, 1H, H2). ^{13}C NMR: δ 56.1 (CH_2O), 101.2 (C5), 108.6 (C8), 108.8 (C10), 111.9 (d, C3′, J C−F = 23.9 Hz), 116.6 (d, C1′, J C−F = 18.4 Hz), 119.9 (d, C5′, J C−F = 3.7 Hz), 130.9 (C6′), 147.6 (C7), 150.2 (C6), 151.5 (C2), 151.9 (d, C4′, J C−F = 11.0 Hz), 155.2 (C9), 157.3 (d, C2′, J C−F = 246.3 Hz), 164.6 (C4). MS−ESI *m/z* 320−322 $[MH]^+$.

A similar procedure was used to prepare **48** and **49**.

***N*-(5-Acetoxy-4-chloro-2-fluorophenyl)-7-hydroxy-6-methoxy-4-quinazolinylamine 50.** A solution of *N*-(5-acetoxy-4-chloro-2-fluorophenyl)-7-benzyloxy-6-methoxy-4-quinazolinylamine **46** (250 mg, 0.54 mmol) in methanol (5 mL), chloroform (5 mL), and DMF (1 mL) was stirred under hydrogen at 1 atm with 5% palladium-on-charcoal catalyst (100 mg) for 4 h. The catalyst was removed by filtration through diatomaceous earth and the solvent removed by evaporation. The residue was dissolved in ethyl acetate, washed with water and brine, and dried ($MgSO_4$). Most of the solvent was removed by evaporation, the mixture was cooled, and hexane added to obtain a solid product that was collected by filtration, washed with hexane/ethyl acetate, and dried to give 170 mg of **50** (45%). ^{1}H NMR: δ 2.37 (s, 3H, CH_3CO), 3.95 (s, 3H, CH_3O), 7.08 (s, 1H, H3′), 7.59 (s, 1H, H8), 7.68 (d, 1H, H6′), 7.78 (s, 1H, H5), 8.34 (s, 1H, H2), 9.48 (s,1H, NH or OH). ^{13}C NMR δ 15.1 (Ar CH_3), 55.6 ($COOCH_3$), 56.0 (OCH_3), 102.2 (C5), 108.0 (C10), 110.0 (C8), 117.5 (d, C3′, J C−F = 22.0 Hz), 121.1 (d, C6′, J C−F = 2.3 Hz), 125.0 (d, C3′, J C−F = 14.7 Hz), 128.4 (d, C6′, J C−F = 7.4 Hz), 144.6 (C6 or C7), 144.7 (C7 or C6), 148.6 (C9), 152.8 (C2), 153.3 (O−C=O), 154.3 (d, C2′, J C−F = 244.6 Hz), 157.0 (C4). MS−ESI *m/z* 394 $[MH]^+$.

7-Benzyloxy-6-methoxy-3-(pivaloyloxymethyl)-3,4-dihydroquinazolin-4-one 51. Sodium hydride (1.44 g of a 60% suspension in mineral oil, 36 mmol) was added in portions over 20 min to a solution of 7-benzyloxy-6-methoxy-3,4-dihydroquinazolin-4-one **40** (8.46 g, 30 mmol) in DMF (70 mL) and the mixture was stirred for 1.5 h. Chloromethyl pivalate (5.65 g, 37.5 mmol) was added dropwise and the mixture stirred for 2 h at ambient temperature. The mixture was diluted with ethyl acetate (100 mL) and poured onto ice−water (400 mL) and 2 N hydrochloric acid (4 mL). The organic layer was separated and the aqueous layer extracted with ethyl acetate, the combined extracts were washed with brine, dried ($MgSO_4$), and the solvent removed by evaporation. The residue was triturated with a mixture of ether and petroleum ether, the solid was collected by filtration, and dried under vacuum to give 10 g of **51** (84%). ^{1}H NMR: δ 1.11 (s, 9H, $C(CH_3)_3$), 3.89 (s, 3H, CH_3O), 5.3 (s, 2H, OCH_2N), 5.9 (s, 2H, CH_2OPh), 7.27 (s, 1H, H8), 7.35 (m, 1H, H14), 7.47 (t, 2H, H13), 7.49 (d, 2H, H12), 7.51 (s, 1H, H5), 8.34 (s, 1H, H2). ^{13}C NMR: δ 26.5 (C−(CH_3)$_3$), 38.3 (C−Me_3), 55.8 (OCH_3), 69.0 (NCH_2O), 70.1 (OCH_3), 105.6 (C5), 109.5 (C8), 114.4 (C10), 127.9 (2 C13), 128.1 (C14), 128.5 (2 C12), 136.1 (C11), 143.6 (C6), 146.3 (C2), 149.1 (C7), 153.8 (C9), 159.0 (C4), 177.0 (O−C=O). MS−ESI *m/z* 397 $[MH]^+$. Anal. ($C_{22}H_{24}N_2O_5 \cdot 0.5\ H_2O$) C, H, N.

7-Hydroxy-6-methoxy-3-(pivaloyloxymethyl)-3,4-dihydroquinazolin-4-one 52. A mixture of 7-benzyloxy-6-methoxy-3-(pivaloyloxymethyl)-3,4-dihydroquinazolin-4-one **51** (7 g, 17.7 mmol) and 10% palladium-on-charcoal catalyst (700 mg) in ethyl acetate (250 mL), DMF (50 mL), methanol (50 mL), and acetic acid (0.7 mL) was stirred under hydrogen at atmospheric pressure for 40 min. The catalyst was removed by filtration and the solvent removed from the filtrate by evaporation. The residue was triturated with ether, collected by filtration, and dried under vacuum to give 4.36 g of **52** (80%). ^{1}H NMR: δ 1.1 (s, 9H, $C(CH_3)_3$), 3.89 (s, 3H, CH_3O), 5.89 (s, 2H, OCH_2N), 7.0 (s, 1H, H8), 7.48 (s, 1H, H5), 8.5 (s, 1H, H2). ^{13}C NMR: δ 26.5 (C−(CH_3)$_3$), 38.3 (C−Me_3), 55.8 (OCH_3), 69.0 (NCH_2O), 106.0 (C5), 111.4 (C8), 113.3 (C10), 143.8 (C7 or C6), 146.1 (C2), 148.5 (C6 or C7), 153.6 (C9), 159.0 (C4), 177.0 (O−C=O). MS−ESI *m/z* 329 $[MNa]^+$. Anal. ($C_{15}H_{18}N_2O_5 \cdot 0.3\ H_2O$, 0.02 DMF) C, H, N.

7-(2-Methoxyethoxy)-6-methoxy-3-(pivaloyloxymethyl)-3,4-dihydroquinazolin-4-one 53. (Procedure L). Diethylazodicarboxylate (1.16 mL, 7.35 mmol) was added dropwise to a solution of 7-hydroxy-6-methoxy-3-(pivaloyloxymethyl)-3,4-dihydroquinazolin-4-one **52** (1.5 g, 4.9 mmol) in dichloromethane (15 mL) containing triphenylphosphine (1.93 g, 7.35 mmol) and 2-methoxyethanol (0.46 mL, 5.88 mmol). After the solution was stirred for 1 h at ambient temperature, the volatiles were removed under vacuum. The solid was purified by column chromatography eluting with ethyl acetate/petroleum ether (1:1) followed by 3:2. After evaporation of the solvent, the solid was triturated with ether, filtered and dried under vacuum to give 1.5 g of **53** (84%). ^{1}H NMR: δ 1.13 (s, 9H, tBu), 3.35 (s, 3H, CH_3O), 3.72 (m, 2H, CH_2O), 3.9 (s, 3H, CH_3O), 4.3 (m, 2H, CH_2O), 5.92 (s, 2H, NCH_2O), 7.2 (s, 1H, H8), 7.51 (s, 1H, H5), 8.37 (s, 1H, H2). ^{13}C NMR: δ 26.5 ($C(CH_3)_3$), 38.1 ($C(CH_3)_3$, 55.7 ($PhOCH_3$), 58.2 (CH_2−O−CH_3), 68.0 (N−CH_2−O), 69.0 (Ph−CH_2−O), 70.0 (CH_2−O−CH_3), 105.4 (C5 or C8), 108.9 (C8 or C5), 114.3 (C10), 143.6 (C6 or

C7), 146.3 (C2), 148.9 (C7 or C6), 154.1 (C9), 159.0 (C4), 177.0 (O−*C*=O). MS−ESI *m/z* 365 $[MH]^+$. Anal. ($C_{18}H_{24}O_6N_2 \cdot 0.18\ H_2O$) C, H, N.

A similar procedure was used to prepare **54**.

6-Methoxy-[7-(2-methoxyethoxy)]-3,4-dihydroquinazolin-4-one 55. (Procedure M). A suspension of 6-methoxy-[7-(2-methoxyethoxy)]-3-[(pivaloyloxy)methyl]-3,4-dihydroquinazolin-4-one **53** (1.35 g, 3.7 mmol) in 7 N ammonia in methanol (50 mL) was stirred at ambient temperature overnight. After removal of the volatiles under vacuum, the residue was triturated with ether, filtered, washed with ether followed by ether/methylene chloride/methanol 7:3:1 and dried under vacuum to give 810 mg of **55** (87%). ^1H NMR: δ 3.35 (s, 3H, CH_3O), 3.75 (m, 2H, CH_2O), 3.9 (s, 3H, CH_3O), 4.24 (m, 2H, CH_2O), 7.15 (s, 1H, H8), 7.46 (s, 1H, H5), 7.99 (s, 1H, H2). ^{13}C NMR: δ 55.6 (Ph−O−*C*H_3), 58.2 (CH_2−O−*C*H_3), 67.9 (Ph−O−*C*H_2), 70.0 (*C*H_2−O−CH_3), 105.0 (C5), 108.7 (C8), 115.6 (C10), 143.8 (C2), 147.7 (C6), 148.5 (C7), 153.6 (C9), 160.0 (C4). MS−ESI *m/z* 251 $[MH]^+$. Anal. ($C_{12}H_{14}O_4N_2 \cdot 0.1\ H_2O$) C, H, N.

A similar procedure was used to prepare **57**.

***N*-(4-Chloro-2-fluorophenyl)-6,7-dihydroxy-4-quinazolinylamine 59.** A mixture of *N*-(4-chloro-2-fluorophenyl)-7-hydroxy-6-methoxy-4-quinazolinylamine **47** (0.5 g., 1.5 mmol) and pyridine hydrochloride (3.6 g, 31 mmol) was melted at 190−220 °C for 1 h. After the solution was cooled, the solid was suspended in water (60 mL) and sonicated for 15 min. The solid was filtered, washed with water followed by ether, and dried under vacuum to give 383 mg of **59** (84%). ^1H NMR: δ 7.42 (s, 2H, H8 and H3'), 7.55 (t, 1H, H6'), 7.65 (d, 1H, H5'), 7.93 (s, 1H, H5), 8.69 (s, 1H, H2), 10.2−10.7 (br s, 1H, NH), 11.0 (bs, 2H, OH). MS−ESI *m/z* 306 $[MH]^+$. Anal. ($C_{14}H_9N_3O_2FCl \cdot 0.75\ HCl$) C, H, N.

2-Amino-5-methoxy-4-nitrobenzoic acid 61. A solution of 2-acetamido-5-methoxy-4-nitrobenzoic acid **60** (21.6 g, 85 mmol) in water (76 mL) and concentrated hydrochloric acid (30.5 mL) was heated at reflux for 3 h. The reaction mixture was cooled to 0 °C, the resulting solid was collected by filtration, washed with water and dried under vacuum to give 16.6 g of **61** (92%). ^1H NMR δ 3.79 (s, 3H, CH_3O), 7.23 (s, 1H, H6), 7.52 (s, 1H, H3), 8.8 (br s, 2H, NH_2).

6-Methoxy-7-nitro-3,4-dihydroquinazolin-4-one 62. A solution of 2-amino-5-methoxy-4-nitrobenzoic acid **61** (16.6 g, 78 mmol) in formamide (250 mL) was heated at reflux for 4.5 h. The reaction mixture was cooled to 0 °C, diluted with water, and the resulting precipitate collected by filtration, washed with water, and dried under vacuum to give 11.56 g of **62** (67%). ^1H NMR (DMSO-d_6, CF_3COOD): δ 4.02 (s, 3H, CH_3O), 7.8 (s, 1H, H8), 8.12 (s, 1H, H2 or H5), 8.18 (s, 1H, H2 or H5). MS−ESI *m/z* 222 $[MH]^+$. Anal. ($C_9H_7N_3O_4 \cdot 0.3\ H_2O$) H, N, C: calcd 47.71, found 47.3.

***N*-7-Amino-(4-chloro-2-fluorophenyl)-6-methoxy-4-quinazolinylamine 66.** A solution of *N*-(4-chloro-2-fluorophenyl)-6-methoxy-7-nitro-4-quinazolinylamine hydrochloride **64** (6 g, 15 mmol) in a mixture of DMF (100 mL) and methanol (600 mL) containing 10% Pd/C (1.8 g) was hydrogenated at 1.3 atm for 2 h. After filtration, the filtrate was evaporated. The solid residue was triturated with a mixture of methylene chloride and ether, filtered, washed with ether, and dried under vacuum to give **66**, which was used without purification in the next stage. MS−ESI *m/z* 319 $[MH]^+$.

***N*-7-Amino-(2-fluoro-5-methoxycarbonyloxy-4-methylphenyl)-6-methoxy-4-quinazolinylamine hydrochloride 67.** A mixture of *N*-(2-fluoro-5-methoxycarbonyloxy-4-methylphenyl)-6-methoxy-7-nitro-4-quinazolinylamine hydrochloride **65** (1.1 g, 25 mmol) and 10% palladium-on-charcoal catalyst (220 mg) in methanol (200 mL) and ethanol (10 mL) was stirred under hydrogen at 2.7 atm for 7 h. The catalyst was removed by filtration through diatomaceous earth, the solvent removed from the filtrate by evaporation and the solid residue washed with ether, collected by filtration, and dried under vacuum to give 930 mg of **67** (91%). ^1H NMR: δ 2.22 (s, 3H, CH_3CO), 3.87 (s, 3H, CH_3O), 4.02 (s, 3H, CH_3O), 6.9 (s, 1H, H8), 7.4−7.5 (m, 2H, H3' and H6'), 7.99 (s, 1H, H5), 8.62 (s, 1H, H2). MS−ESI *m/z* 372 $[MH]^+$.

***N*-(2-Fluoro-5-methoxycarbonyloxy-4-methylphenyl)-6-methoxy-7-methoxyacetamido-4-quinazolinylamine 68.** Methoxyacetyl chloride (62 μL, 0.68 mmol) was added dropwise to a solution of *N*-7-amino-(2-fluoro-5-methoxycarbonyloxy-4-methylphenyl)-6-methoxy-4-quinazolinylamine hydrochloride **67** (215 mg, 0.52 mmol) in methylene chloride (5 mL) and pyridine (1.5 mL) at 0 °C and the mixture stirred for 2 h at 0 °C. Further methoxyacetyl chloride (14 μL, 0.15 mmol) was added and the mixture stirred for further 20 min at 0 °C. The reaction mixture was partitioned between ethyl acetate and water and the aqueous layer adjusted to pH = 9 with saturated aqueous sodium bicarbonate solution. The organic layer was separated, washed with brine, dried ($MgSO_4$) and the solvent removed by evaporation. The residue was purified by column chromatography eluting with methylene chloride/acetonitrile/methanol (60:38:2) to give 175 mg of **68** (75%). ^1H NMR: δ 2.21 (s, 3H, CH_3CO), 3.47 (s, 3H, CH_3O), 3.87 (s, 2H, CH_2O), 4.07 (s, 3H, CH_3O), 4.15 (s, 3H, CH_3OPh), 7.35 (d, 1H, H3'), 7.45 (d, 1H, H6'), 7.96 (s, 1H, H8), 8.40 (s, 1H, H5), 8.65 (s, 1H, H2), 9.28 (s, 1H, NH), 9.65 (s, 1H, NH).

7-Fluoro-3,4-dihydroquinazolin-4-one 69. A solution of 2-amino-4-fluorobenzoic acid (3 g, 19.3 mmol) in formamide (30 mL) was heated at 150 °C for 6 h. The reaction mixture was poured onto ice−water (1:1) (250 mL). The precipitated solid was collected by filtration, washed with water, and dried to give 2.6 g of **69**[27] (82%). ^1H NMR (DMSO-d_6, CF_3COOD): δ 7.42 (m, 1H, H6), 7.48 (dd, 1H, H8), 8.22, (dd, 1H, H5), 8.4 (s, 1H, H2). MS−EI *m/z* 164 $[M]^+$. Anal. ($C_8H_5FN_2O$) C, H, N.

7-(2-Methoxyethoxy)-3,4-dihydroquinazolin-4-one 70. Sodium (400 mg, 17 mmol) was added carefully to 2-methoxyethanol (10 mL) and the mixture heated at reflux for 30 min. 7-Fluoro-3,4-dihydroquinazolin-4-one **69**[27] (750 mg, 4.57 mmol) was added to the resulting solution and the mixture heated at reflux for 15 h. The mixture was cooled and poured into water (250 mL). The mixture was acidified to pH = 4 with concentrated hydrochloric acid. The resulting solid product was collected by filtration, washed with water and then with ether, and dried under vacuum to give 580 mg of **70** (58%). ^1H NMR (DMSO-d_6, CF_3COOD): δ 3.95 (s, 3H, CH_3O), 4.4 (t, 2H, CH_2O), 4.95 (t, 2H, CH_2O), 7.85 (d, 1H, H8), 7.95 (dd, 1H, H6), 8.75 (d, 1H, H5), 9.62 (s, 1H, H2). MS−EI *m/z* 220$[M]^+$. Anal. ($C_{11}H_{12}N_2O_3$) C, H, N.

5-Methoxy-4-(2-methoxyethoxy)-2-nitroacetophenone 75. 3-Methoxy-4-(2-methoxyethoxy)acetophenone **74** (18.1 g, 80 mmol) was added in portions over 50 min to a solution of nitric acid (163 mL, 69.5%) and cooled to 2 °C. After the solution was stirred for 2 h at ambient temperature, the reaction mixture was poured onto ice and extracted with ethyl acetate. The organic layer was washed with water and brine, dried ($MgSO_4$), and the solvent evaporated. The residue was purified by flash chromatography using methylene chloride/ethyl acetate (95:5) as eluent to give 17.4 g of **75** (80%). Mp 120−124 °C. ^1H NMR ($CDCl_3$): δ 2.5 (s, 3H, CH_3CO), 3.45 (s, 3H, CH_3OCH_2), 3.8 (t, 2H, CH_2OCH_3), 3.95 (s, 3H, CH_3O), 4.25 (t, 2H, CH_2OAr), 6.75 (s, 1H, H6), 7.7 (s, 1H, H3).^{13}C NMR: δ 29.9 (CO−*C*H_3), 56.6 (Ph−O−*C*H_3), 58.1 (CH_2−O−*C*H_3), 68.4 (Ph−O−*C*H_2), 69.9 (CH_3−O−*C*H_2), 108.1 (C8), 109.8 (C5), 131.2 (C10), 138.3 (C9), 148.5 (C6 or C7), 153.2 (C7 or C6), 199.3 (C=O). MS−EI *m/z* 269 $[M]^+$. Anal. ($C_{12}H_{15}NO_6$) C, H, N.

2-Amino-5-methoxy-4-(2-methoxyethoxy)acetophenone 76. Iron powder (10 g, 180 mmol) was added in portions to a solution of 2-nitro-4-(2-methoxyethoxy)-5-methoxyacetophenone **75** (17.3 g, 64 mmol) in acetic acid (80 mL) and heated at 100 °C. After the solution was stirred for 30 min at 100 °C, the mixture was cooled and water (20 mL) was added. The mixture was extracted with ethyl acetate, the combined extracts were washed with water, saturated sodium carbonate solution, and brine and then dried ($MgSO_4$), and the solvent was evaporated. The residue was purified by flash chromatography using methylene chloride/ethyl acetate (8:2 followed by 75:25) as eluent to give 12.52 g of **76** (81%). Mp 99−101 °C. ^1H NMR ($CDCl_3$): δ 2.52 (s, 3H, CH_3CO), 3.45 (s, 3H, CH_3OCH_2), 3.8 (t, 2H, CH_2O), 3.85 (s, 3H, CH_3O), 4.15 (t, 2H,

CH_2O), 6.12 (s, 1H), 6.22 (bs, 1H), 7.12 (s, 1H). MS–EI *m/z* 239 $[M]^+$. Anal. ($C_{12}H_{17}NO_4$) C, H, N.

6-Methoxy-7-(2-methoxyethoxy)-1,4-dihydroquinolin-4-one 77. A mixture of 4-methoxy-3-(2-methoxyethoxy)aniline **81** (5 g, 25.3 mmol) and diethylethoxymethylenemalonate (6 mL, 30 mmol) was heated at 110 °C for 30 min. Diphenyl ether (5 mL) was added and the mixture was heated at 240 °C for 6 h. The mixture was allowed to cool and diluted with petroleum ether. The resulting solid was collected by filtration and purified by reverse phase chromatography on a Diaion (trade mark of Mitsubishi) HP20SS resin column eluting with acetonitrile/water (40:60) to give 500 mg of **77** (8%).^{1}H NMR: δ 3.35 (s, 3H, CH_3O), 3.75 (dd, 2H, CH_2O), 3.85 (s, 3H, CH_3O–Ph), 4.18 (dd, 2H, CH_2O), 5.95 (d, 1H, H2), 7.0 (s, 1H, H5), 7.48 (s, 1H, H8), 7.78 (d, 1H, H3). ^{13}C NMR: δ 55.4 (Ph–O–*C*H_3), 58.2 (CH_2–O–*C*H_3), 67.7 (Ph–O–*C*H_2), 70.0 (CH_3–O–*C*H_2), 100.0 (C8), 104.3 (C5), 107.7 (C10), 119.8 (C10), 137.9 (C2), 146.5 (C6), 151.9 (C7), 175.6 (C4). MS–ESI *m/z* 250 $[MH]^+$.

4-Methoxy-3-(2-methoxyethoxy)nitrobenzene 80. A mixture of 2-methoxy-5-nitrophenol **79** (6 g, 35 mmol), 2-bromoethyl methyl ether (4 mL, 40 mmol), potassium carbonate (5.8 g, 40 mmol), and potassium iodide (0.5 g) in DMF (50 mL) was heated at 80 °C for 1 h. The mixture was allowed to cool and then poured into water (400 mL). The resulting precipitate was collected by filtration, washed with water, and dried under vacuum to give 7.75 g of **80** (98%). ^{1}H NMR ($CDCl_3$): δ 3.46 (s, 3H, CH_3O), 3.82 (t, 2H, CH_2O), 3.96 (s, 3H, CH_3OPh), 4.25 (t, 2H, CH_2O), 6.91 (d, 1H, H5), 7.79 (d, 1H, H2), 7.92 (dd, 1H, H6). ^{13}C NMR: δ 56.2 (Ph–O–*C*H_3), 58.1 (CH_2–O–*C*H_3), 68.1 (Ph–O–*C*H_2), 70.1 (*C*H_2–O–CH_3), 107.3 (C8), 111.0 (C5), 117.7 (C10), 140.6 (C9), 147.7 (C7), 154.7 (C6). MS–ESI *m/z* 227 $[MH]^+$.

4-Methoxy-3-(2-methoxyethoxy)aniline 81. A mixture of 4-methoxy-3-(2-methoxyethoxy)nitrobenzene **80** (7 g, 30 mmol) and 10% palladium on charcoal catalyst (1.4 g) in ethyl acetate (70 mL) was stirred under hydrogen at 3.3 atm pressure for 1 h. The catalyst was removed by filtration through diatomaceous earth and the solvent removed by evaporation. The solid residue was suspended in ethyl acetate, collected by filtration, and dried under vacuum to give 6.1 g of **81** (100%). ^{1}H NMR ($CDCl_3$): δ 3.4 (s, 3H, CH_3O), 3.75 (t, 2H, CH_2O), 3.8 (s, 3H, CH_3OPh), 4.12 (t, 2H, CH_2O), 6.24 (dd, 1H, H6), 6.34 (d, 1H, H2), 6.7 (d, 1H, H5). ^{13}C NMR: δ 56.6 (Ph–O–*C*H_3), 58.1 (CH_2–O–*C*H_3), 67.3 (Ph–O–*C*H_2), 70.4 (*C*H_2–O–CH_3), 101.1 (C8), 105.5 (C10), 114.9 (C5), 140.2 (C9), 143.3 (C7), 148.9 (C6). MS–ESI *m/z* 197 $[MH]^+$.

4-Hydroxy-6-methoxy-7-(2-methoxyethoxy)cinnoline 82. A solution of sodium nitrite (3.9 g, 56 mmol) in water (5 mL) was added dropwise, to a solution of 2-amino-5-methoxy-4-(2-methoxyethoxy)acetophenone **76** (12.18 g, 50 mmol) in acetic acid (180 mL) and sulfuric acid (30 mL). After the solution was stirred for 90 min at 80 °C, the solution was concentrated to half its original volume and poured into ether (800 mL). The solid was collected by filtration and suspended in water (400 mL). After the pH was adjusted to 7.6 with 2 N aqueous sodium hydroxide solution, the solid was filtered off and washed with ether to give 8 g of **82** (62%). Mp 232–234 °C. ^{1}H NMR (DMSO-d_6, CF_3COOD): δ 3.35 (s, 3H, CH_3O), 3.75 (t, 2H, CH_2OCH_3), 3.9 (s, 3H, CH_3O), 4.2 (t, 2H, CH_2OAr), 6.95 (s, 1H, H3), 7.35 (s, 1H, H8), 7.65 (s, 1H, H5). ^{13}C NMR: δ 55.7 (Ph-O-*C*H_3), 58.2 (CH_2–O–*C*H_3), 67.9 (Ph–O–*C*H_2), 69.9 (CH_3–O–*C*H_2), 97.1 (C8), 102.1 (C5), 117.8 (C10), 137.4 (C6), 138.9 (C3), 148.3 (C9), 153.8 (C7), 168.5 (C4). MS–EI *m/z* 250-$[M]^+$. Anal. ($C_{12}H_{14}N_2O_4$) C, H, N.

4-Chloro-6-methoxy-7-(2-methoxyethoxy)cinnoline 83. A solution of 4-hydroxy-6-methoxy-7-(2-methoxyethoxy)cinnoline **82** (7.8 g, 31 mmol) in thionyl chloride (130 mL) containing DMF (0.8 mL) was stirred at 80 °C for 2 h. After dilution with toluene, the mixture was evaporated to dryness. The resulting solid was filtered off, washed with ether, and then dissolved in ethyl acetate. The ethyl acetate solution was washed with saturated aqueous sodium bicarbonate solution, brine, dried ($MgSO_4$), and the solvent evaporated. The residue was purified by flash chromatography using methylene chloride/ethyl acetate (1:9) as eluent to give 6.2 g of **83** (74%). Mp 171–173 °C. ^{1}H NMR ($CDCl_3$): δ 3.52 (s, 3H, CH_3OCH_2), 3.9 (t, 2H, CH_2-OCH_3), 4.1 (s, 3H, OCH_3), 4.4 (s, 2H, CH_2OAr), 7.75 (s, 1H, H5), 7.23 (s, 1H, H8), 9.15 (s, 1H, H3). ^{13}C NMR: δ 56.5 (*C*H_3O), 58.2 (CH_2O*C*H_3), 68.4 (Ph*C*H2O), 69.9 (CH_3O*C*H_2), 99.1 (C8), 107.2 (C5), 121.0 (C4), 131.3 (C10), 143.1 (C3), 148.4 (C9), 153.0 (C6 or C7), 154.6 (C6 or C4). MS–EI *m/z* 268 $[M]^+$. Anal. ($C_{12}H_{13}N_2O_3Cl$) C, H, N.

4-Fluoro-2-methylphenyl methyl carbonate 84. Methyl chloroformate (6.8 mL, 88 mmol) was added over 30 min to a solution of 4-fluoro-2-methylphenol (10 g, 79 mmol) in 6% aqueous sodium hydroxide solution at 0 °C. The mixture was stirred for 2 h, then extracted with ethyl acetate (100 mL). The ethyl acetate extract was washed with water (100 mL) and dried ($MgSO_4$) and the solvent removed by evaporation to give 11.4 g of **84** (78%).^{1}H NMR: δ 2.14 (s, 3H, CH_3), 3.81 (s, 3H, CH_3O), 7.05 (m, 1H), 7.1–7.25 (m, 2H).

4-Fluoro-2-methyl-5-nitrophenol 85. A mixture of concentrated nitric acid (6 mL) and concentrated sulfuric acid (6 mL) was added slowly to a solution of 4-fluoro-2-methylphenyl methyl carbonate **84** (11.34 g, 62 mmol) in concentrated sulfuric acid (6 mL) such that the temperature of the mixture was kept below 50 °C. The mixture was stirred for 2 h, added to ice–water (1:1) and the precipitated product collected by filtration. The crude product was purified by chromatography on silica eluting with methylene chloride/hexane progressing through increasingly polar mixtures to methanol/methylene chloride (1:19) to give 2.5 g of **85** (22%). ^{1}H NMR (DMSO-d_6, CD_3COOD): δ 2.31 (s, 3H, CH_3), 7.38 (d, 1H, H3), 7.58 (d, 1H, H6). MS–ESI *m/z* 171 $[MH]^+$.

2-Fluoro-5-hydroxy-4-methylaniline 86. A mixture of 4-fluoro-2-methyl-5-nitrophenol **85** (2.1 g, 13 mmol), iron powder (1 g, 18 mmol) and iron(II)sulfate (1.5 g, 10 mmol) in water (40 mL) was heated at reflux for 4 h. The mixture was allowed to cool, neutralized with 2 N aqueous sodium hydroxide, and extracted with ethyl acetate (100 mL). The ethyl acetate extract was dried ($MgSO_4$) and the solvent removed by evaporation to give 0.8 g of **86** (47%). ^{1}H NMR: δ 1.94 (s, 3H, CH_3), 4.67 (s, 2H, NH_2), 6.22 (d, 1H, H3 or H6), 6.65 (d, 1H, H3 or H6), 8.68 (s, 1H, OH). MS–ESI *m/z* 142 $[MH]^+$.

2-Chloro-4-fluoro-methoxycarbonyloxybenzene 87. To a solution of 0.5 N aqueous sodium hydroxide cooled at 5 °C was added 2-chloro-4-fluorophenol (29.3 g, 0.2 mol), followed by dropwise addition of methylchloroformate (23.6 g, 0.25 mol), while keeping the temperature below 5–10 °C. After the solution was stirred for 15 min at this temperature, the solid was filtered, washed with water and dried under vacuum to give 40.5 g of **87** (99%). Mp 73–74 °C. ^{1}H NMR ($CDCl_3$): δ 3.9 (s, 3H, CH_3O), 7.05 (dt, 1H, H6), 7.2 (m, 2H, H5 and H3). MS–EI *m/z* 204 $[M]^+$. Anal. ($C_8H_6ClFO_3$) C, H.

2-Chloro-4-fluoro-5-nitro-methoxycarbonyloxybenzene 88. To a suspension of 2-chloro-4-fluoro-methoxycarbonyloxybenzene **87** (40 g, 0.2 mol) in concentrated sulfuric acid (15 mL) was added a mixture of sulfuric acid (15 mL) and nitric acid 70% (15 mL), dropwise to keep the temperature below 30 °C. The mixture was stirred for 30 min at this temperature after completion of the addition. The mixture was poured onto a mixture of ice–water. The orange solid was filtered, washed with water, and dried under vacuum. The solid was dissolved in ether and the ethereal solution was washed with water, brine, dried ($MgSO_4$), filtered, and evaporated to give 41 g of **88** (82%). Mp 59–60 °C. ^{1}H NMR ($CDCl_3$): δ 3.98 (s, 3H, CH_3O), 7.45 (d, 1H, H3), 8.05 (d, 1H, H6). MS-EI *m/z* 249 $[M]^+$. Anal. ($C_8H_5ClFNO_5$) C, H, N.

5-Amino-2-chloro-4-fluoro-methoxycarbonyloxybenzene 89. A solution of 2-chloro-4-fluoro-5-nitro-methoxycarbonyloxybenzene **88** (20 g, 80 mmol) in ethanol (300 mL) containing platinum oxide (200 mg) was hydrogenated for 3 h and ethyl acetate (200 mL) was added. Hydrogenation was continued for 2 h, and the mixture was filtered. The solvent was removed by evaporation and the oily residue triturated with ether. The resulting solid was filtered, washed with ether,

and dried under vacuum to give 15 g of **89** (82%). Mp 85–86 °C. ^{1}H NMR ($CDCl_3$): δ 3.92 (s, 3H, CH_3O), 6.63 (d, 1H, H6), 7.08 (d, 1H, H3). MS–EI *m/z* 219 $[M]^+$. Anal. ($C_8H_7ClFNO_3$) C, H, N.

2-[*N*-Methyl-*N*-(4-pyridyl)]aminoethanol 90. A solution of 4-chloropyridine hydrochloride (4.5 g, 30 mmol) and 1-methyl-1-(hydroxyethyl)amine (2.25 g, 30 mmol) in 3-methyl-1-butanol (50 mL) containing $NaHCO_3$ (7.56 g, 90 mmol) was refluxed for 2 days. After removal of the solvant under vacuum, the residue was triturated with ethyl acetate. The solid was filtered off and washed with more ethyl acetate. The ethyl acetate layers were combined and evaporated. The resulting brown oil was purified by chromatography on neutral alumina eluting with methylene chloride–methanol (96:4) to give 300 mg of **90** (6%). Mp 87–88 °C. ^{1}H NMR ($CDCl_3$): δ 3.05 (s, 3H, NCH_3), 3.53 (t, 2H, CH_2OH), 3.83 (t, 2H, CH_2N), 6.5 (d, 2H, pyridine C=CH), 8.15 (d, 2H, pyridine CH=N–CH). MS–EI *m/z* 152 $[M]^+$. Anal. ($C_8H_{12}N_2O \cdot 0.1$ MeOH) C, H, N.

Biological Evaluation. IC_{50} reported are average figures of 3 to 5 measurements, depending on the compound potency.

(a) In Vitro Receptor Tyrosine Kinase Inhibition Test. This assay determined the ability of a test compound to inhibit tyrosine kinase activity.

Flt-1 (Genbank accession number × 51602), KDR (Genbank accession number L04947), and FGFR1 (Genbank accession number × 51803) cytoplasmic domains (2, 16, and 2 amino acids after the transmembrane region to the C-terminus, respectively), were isolated by PCR from a human placental cDNA library (Clontech Cat no. HL1008b). In each case, a methionine codon was added to the 5′-end, and coding regions were flanked by *Bam*H1 restriction sites to enable cloning into the baculovirus expression vector pACYM1. Recombinant proteins were expressed in Spodoptera frugiperdera 21 (Sf 21) insect cells. For KDR and FGFR1 receptor tyrosine kinases, 3 × 10^7 Sf21 cells were infected with plaque-pure recombinant virus at a multiplicity of infection of 3 (175 cm^3 flask). Flt-1 expression was achieved by infecting 2 × 10^6 Sf9 insect cells at a multiplicity of infection of 1 (5 L bioreactor). Cells were harvested after 48 h, washed with ice cold phosphate buffered saline (PBS), and resuspended in an ice cold HNTG/PMSF solution (20 mM Hepes, pH 7.5, 150 mM sodium chloride, 10% v/v glycerol, 1% v/v Triton × 100, 1.5 mM magnesium chloride, 1 mM ethylene glycol–bis(baminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF)), using 1 mL of HNTG/PMSF per 10^6 cells. The suspension was centrifuged for 10 min at 13 000 rpm at 4 °C, and the supernatant (enzyme stock) removed and stored in aliquots at −70 °C. Each new batch of stock enzyme was titrated in the assay following dilution with enzyme diluent (100 mM Hepes pH 7.4, 0.2 mM Na_3VO_4, 0.1% v/v Triton × 100, 0.2 mM DTT). For a typical batch, stock enzyme was diluted 1 in 2000 with enzyme diluent and 50 μL of dilute enzyme used per well.

A poly(glu, ala, tyr) 6:3:1 random copolymer (Sigma, Poole, UK) was used as a tyrosine containing substrate solution. The substrate was stored as a 1 mg/mL stock in PBS at −20 °C and diluted 1 in 500 with PBS in order to coat 96 well plates (100 μL/well). Plates were coated on the day prior to assay, sealed with adhesive seals, and stored overnight at 4 °C. On the day of the assay, the substrate solution was discarded and the assay plate wells were washed once with PBST (PBS containing 0.05% v/v Tween 20) and once with Hepes buffer (50 mM, pH 7.4).

Test compounds were diluted with 10% dimethylsulfoxide (DMSO) de-ionized water and 25 μL volumes transferred to wells in the washed assay plates. Manganese chloride solution (40 mM) containing 8 μM ATP was then added (25 μL) to all test wells. Control and blank wells, containing compound diluent and manganese chloride solution with and without ATP, respectively, were also included to determine the dynamic range of the assay. Freshly diluted enzyme (50 μL) was added to each well, and the plates incubated at room temperature for 20 min. The liquid was then discarded and the wells were washed twice with PBST. Mouse IgG anti-phosphotyrosine antibody (Upstate Biotechnology Inc., Lake Placid, USA) diluted 1:6000 with PBST containing 0.5% (w/v) bovine serum albumin (BSA) was added (100 μL/well), and the plates incubated for 1h at room temperature before discarding the liquid and washing the wells twice with PBST. Horseradish peroxidase (HRP)-linked sheep anti-mouse Ig antibody (Amersham, Little Chalfont, UK) diluted 1:500 with PBST containing 0.5% (w/v) BSA, was then added (100 μL/well) and the plates incubated for a further 1 h at room temperature before discarding the liquid and washing the wells twice with PBST. A 1 mg/mL solution of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (Boehringer, Lewes, UK) was freshly prepared in 50 mM phosphate-citrate buffer (pH5.0) containing 0.03% (w/v) sodium perborate, and 100 μL added to each well. Plates were then incubated for 20–60 min at room temperature until the optical density value of control wells measured at 405 nm was approximately 1.0. IC_{50} values for compound enzyme inhibition were interpolated using Microcal Origin following subtraction of blank values.

The exact enzyme concentration of the different preparations of partially purified enzyme isolated from the insect cell lysate is not known. The precise state of phosphorylation of these partially purified enzymes is also unknown. However, the degree of phosphorylation of several batches of enzyme protein, prepared by the method described, was checked by western blotting, using anti-phosphotyrosine antibody, and this did not appear to grossly influence the activity of the enzyme.

(b) In Vitro HUVEC Proliferation Assay. HUVEC cells were isolated in MCDB 131 (Gibco BRL) + 7.5% v/v foetal calf serum (FCS) and were plated out (at passage 2 to 8), in MCDB 131 + 2% v/v FCS + 3 μg/mL heparin + 1 μg/mL hydrocortisone, at a concentration of 1000 cells/well in 96 well plates. After a minimum of 4 h they were dosed with the appropriate growth factor (i.e., $VEGF_{165}$ 3 ng/mL or b-FGF 0.3ng/mL) and compound. The cultures were then incubated for 4 days at 37 °C with 7.5% CO_2. On day 4, the cultures were pulsed with 1 μCi/well of tritiated thymidine (Amersham product TRA 61) and incubated for 4 h. The cells were harvested using a 96-well plate harvester (Tomtek) and then assayed for incorporation of tritium with a Beta plate counter. Incorporation of radioactivity into cells, expressed as cpm, was used to measure inhibition of growth factor-stimulated cell proliferation by compounds.

(c) In Vivo Rat Uterine Oedema Assay. The assay was performed in groups of newly weaned, 20- to 22-day old female rats. One group received dosing vehicles only and served as the "untreated" controls. All other groups were treated with a single subcutaneous dose of oestradiol benzoate (2.5 μg/rat) in arachis oil. One group received only this treatment and served as the "oestrogen alone" controls. To the remaining groups, test compounds were administered orally 18 h and 1 h prior to the administration of oestradiol benzoate. The compounds were administered as ball-milled suspensions in 0.1% aqueous polysorbate 80. Five hours after the administration of oestradiol benzoate, the rats were humanely killed and their uteri were dissected, blotted, and weighed. The increase in uterine weight in groups treated with test compound and oestradiol benzoate and with oestradiol benzoate alone was compared using a Student T test. Inhibition of the effect of oestradiol benzoate was considered significant when $p < 0.05$.

The VEGF m-Ab used as VEGF scavenger in the uterine oedema assay was purchased from R&D Systems.

(d) Mouse Strain and Dosing Methodology. Female Swiss mice were used in plasma pharmacokinetic studies, while tumor xenograft experiments used female athymic (nu/nu genotype) Swiss mice maintained in negative pressure isolators (PFI Systems Ltd, Oxon, UK). Mice were bred at Alderley Park, housed in a barrier facility with 12 h light/dark cycles and provided with sterilized food and water ad libitum. All procedures were performed on mice of at least 8 weeks of age, within a weight range of 27–35 g. In all studies, compounds were suspended in a 1% (v/v) solution of polyoxy-

ethylene (20) sorbitan mono-oleate in distilled water, and dosed by oral gavage at 0.1 mL/10 g body weight.

(e) Plasma Pharmacokinetic Assay. Unless specified, all reagents were of Analar or HPLC grade and obtained from Fisher Scientific Ltd. (Loughborough, UK). Compounds were administered to groups of three mice, and blood collected 6 h after dosing by pooling samples containing a given compound into a 1.5 mL lithium heparin tube. Samples were immediately centrifuged (16000*g*, 10 min), plasma removed by aspiration into 1.5 mL microcentrifuge tubes, and duplicate 200 μL aliquots removed for analysis. Additional plasma aliquots containing either no compound (as a control), or a series of compound standards (serially diluted 1:2 to give a range from 5.8 to 0.09 μM) were also prepared in duplicate. Acetonitrile (400 μL) was added to each sample aliquot, while gently vortexing to precipitate plasma protein. Following additional vortexing (15 s) tubes were centrifuged (16000*g*, 30 s), and 550 μL of supernatant removed and diluted with deionized water (250 μL).

Plasma concentrations were determined by reverse-phase HPLC with UV detection. Samples were analyzed using a Constametric 3000 pump and a Spectromonitor D variable wavelength UV detector (LDC Milton Roy, Staffs, UK), with an ISS-101 autosampler (Perkin-Elmer, Uberligen, Germany). Pump control and data acquisition were performed using EZ Chrom Chromatography software (Version 6.6, Scientific Software Inc., San Ramon, CA) and chromatographic separation achieved using a Columbus C18 analytical column (3 μm; 100 × 4.6 mm I.D.), preceded by a Columbus C18 guard cartridge (3 μm; 10 × 3.2 mm I.D.) (Phenomenex Ltd., Macclesfield, UK). A mobile phase of 70% (v/v) methanol in 0.1 M ammonium acetate (prepared in double distilled water) was degassed with a stream of helium (15 min) prior to use. Samples (150 μL injection volume) were eluted isocratically at a flow rate of 1 mL/min, under ambient temperature, and compounds detected within a wavelength range of 335−340 nm. Calibration curves were constructed using peak height and concentrations of unknowns interpolated accordingly. Results were reported as the mean of duplicate samples.

(f) Human Tumor Xenograft Test. The human lung carcinoma cell line Calu-6 was obtained from the American Type Culture Collection (Manassas, VA). All cell culture reagents, unless otherwise specified, were obtained from Life Technologies, Paisley, UK). Cells were maintained as an exponentially growing monolayer in Minimal Essential Medium with Earles' salts, supplemented with 1 mM sodium pyruvate, 1% nonessential amino acids, 10% FCS (Labtech International, Ringmer, UK) and 2 mM L-glutamine (Sigma Chemical Co., Poole, UK). The cell line was found to be negative for microplasma in culture, and for 15 types of virus when screened in a mouse antibody production test (Central Toxicology Laboratories, Alderley Park, UK) prior to routine use in vivo.

Calu-6 tumor xenografts were established in the hind flank of mice by s.c. injection of 1×10^6 cells, in 100 μL of 50% (v/v) Matrigel (Fred Baker, Liverpool, UK) in serum free media. Ten days after cell implantation, mice were divided into groups with tumor sizes ranging from 0.33 to 0.54 cm^3, and received either compound or vehicle once daily for 21 days. Tumor volume was assessed twice weekly by bilateral vernier caliper measurement, using the formula (length × width) × $\sqrt{}$(length × width) × (π/6), where length was the longest diameter across the tumor and width the corresponding perpendicular. Growth inhibition from the start of treatment was calculated by comparison of the mean change in tumor volume for the control and treated group, and statistical significance evaluated using a one-tailed t-test.

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Supporting Information Available: The complete experimental procedures for the synthesis of **3−10,12, 14, 17−19, 21, 22, 25, 26, 28, 30, 35, 41, 43−45, 48, 49, 54, 56−58, 63−65, 71, 72, 74, 78.** This material is available free of charge via the Internet at http://pubs.acs.org.

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Endothelial Precursor Cells As a Model of Tumor Endothelium: Characterization and Comparison with Mature Endothelial Cells

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ABSTRACT

Human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC) have been the standards for cell-based assays in the field of angiogenesis research and in antiangiogenic drug discovery. These normal mature endothelial cells may not be most representative of human tumor endothelial cells. Human AC133+/CD34+ bone marrow progenitor cells were established in cell culture media containing vascular endothelial growth factor, basic fibroblast growth factor (bFGF), and heparin to drive differentiation toward the endothelial phenotype. The resulting cells designated endothelial precursor cells (EPC) have many of the same functional properties as mature endothelial cells represented by HUVEC and HMVEC. By SAGE analysis, the genes expressed by EPC are more similar to the genes expressed by endothelial cells isolated from fresh surgical specimens of human tumors than are the genes expressed by HUVEC and HMVEC. Analysis of several cell surface markers by flow cytometry showed that EPC, HUVEC, and HMVEC have similar expression of P1H12, vascular endothelial growth factor 2, and endoglin but that EPC have much lower expression of ICAM1, ICAM2, VCAM1, and thrombomodulin than do HUVEC and HMVEC. The EPC generated can form tubes/networks on Matrigel, migrate through porous membranes, and invade through thin layers of Matrigel similarly to HUVEC and HMVEC. However, in a coculture assay using human SKOV3 ovarian cancer cell clusters in collagen as a stimulus for invasion through Matrigel, EPC were able to invade into the malignant cell cluster, whereas HMVEC were not able to invade the malignant cell cluster. *In vivo*, a Matrigel plug assay where human EPC were suspended in the Matrigel allowed tube/network formation by human EPC to be carried out in a murine host. EPC may be a better model of human tumor endothelial cells than HUVEC and HMVEC and, thus, may provide an improved cell-based model for second generation antineoplastic antiangiogenic drug discovery.

INTRODUCTION

The importance of normal cells and tissues to support the growth of tumors has been recognized for centuries. The observations of Van der Kolk (1), Jones (2), and Paget (3) documented this knowledge in the clinical science literature. Algire and Chalkey (4) reported that host vascular reactions could be elicited by growing tumors and described in detail the extent and tumor-specific nature of the induction of host capillaries by transplanted tumors. The central hypothesis of Algire and Chalkey was that vascular induction by solid tumors may be the major distinguishing factor leading to tumor growth beyond normal tissue control levels. By the late 1960s, Folkman *et al.* (5–7) had begun the search for a tumor angiogenesis factor, and in 1971, Folkman proposed "antiangiogenesis" as a means of holding tumors in a nonvascularized dormant state (8).

Investigators working in embryogenesis distinguished between angiogenesis, vessels arising from sprouts on existing vessels, and vasculogenesis, vessels arising from endothelial progenitor cells (angioblasts; Refs. 9 and 10). The abnormality of tumor vasculature and value of working with fresh, noncultured live endothelial cells isolated from solid tumors were recognized by cancer researchers (11), and the role of endothelial precursor cells from bone marrow was recognized by researchers studying mammalian development (12, 13). Asahara *et al.* (14–16) isolated putative endothelial precursor cells or angioblasts from human peripheral blood by magnetic bead selection and described a role for these cells in postnatal vasculogenesis and pathological neovascularization. It was later shown that the recruitment of the progenitor cells from the bone marrow requires that activity of matrix metalloproteinase-9 mediation of the release of Kit-ligand (17). Studies in allogeneic bone marrow transplant recipients confirmed that circulating endothelial precursor cells or angioblasts in peripheral blood originated from the bone marrow (18). Gehling *et al.* (19) identified CD34+/AC133+ progenitor cells from bone marrow as a subpopulation of cells that *in vitro* could differentiate into endothelial cells.

Recent studies have formally tied circulating endothelial precursor cells to the development of tumor vasculature (20–24). Gill *et al.* (22) found that in patients with vascular insult secondary to burns or coronary artery bypass grafting, there was a ~50-fold increase in circulating endothelial precursor cells within the first 6–12 h after injury and lasting 48–72 h in parallel with the plasma levels of VEGF. A similar pattern of mobilization of endothelial precursor cells from the bone marrow was observed in mice after injection with VEGF. Using the Id1+/−Id3−/− mutant mouse that has impaired tumor vascular growth, it was shown that transplantation with wild-type bone marrow or with VEGF-mobilized bone marrow stem cells allowed recruitment of endothelial precursor cells sufficient to support tumor growth (23). De Bont *et al.* (24) found that when NOD/SCID mice bearing human Daudi non-Hodgkin's lymphoma xenografts were injected i.v. with human CD34+ hematopoietic stem cells or angioblasts that the injected human CD34+ cells homed to the tumor and differentiated along the endothelial lineage.

AC133+ multipotent human bone marrow progenitor cells exposed to VEGF in cell culture differentiate into CD34+/VE−cadherin+/VEGFR2+ cells or angioblasts (25). On maintenance in cell culture, these cells will continue to differentiate toward a more mature endothelial phenotype. When human AC133+ progenitor cells were injected i.v. into NOD/SCID mice bearing s.c. murine Lewis lung carcinoma, these cells contributed to the developing tumor vasculature. Additional support for the notion that tumor vasculature arises, in part, through the process of vasculogenesis comes from studies in which murine endothelial precursor cells from bone marrow, peripheral blood, and tumor-infiltrating cells were isolated from mice bearing human breast carcinoma xenografts (26). The number of endothelial precursor cells was elevated in the tumor-bearing mice compared with normal mice. There was a significant number of endothelial precursor cells found in the human breast carcinoma xenografts, and maturation and proliferation of these cells in the tumors were evident. Recently, it was reported that NOD/SCID mice transplanted with human bone marrow and bearing human Namalwa or Granta 519 Burkitt's lymphoma xenografts had a 7-fold increase in circulating endothelial precursor cells compared with nontumor-bear-

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ing mice (27). Continuous infusion of endostatin into the tumor-bearing mice resulted in inhibition of the mobilization of endothelial precursor cell from the bone marrow.

To date, most research directed toward the development of antiangiogenic anticancer agents has used the cells readily at hand, primarily HUVECs and human microvascular endothelial cells, as the cell-based models of the tumor endothelium (28). It may be that human endothelial precursor cells are a more representative model of tumor endothelium. The current report compares endothelial precursor cells generated in cell culture to HMVEC[2] and HUVEC grown in culture by the expression of molecular markers and behavior in functional assays.

MATERIALS AND METHODS

Cell Culture. CD34+/AC133+ progenitor cells from human bone marrow cells, HUVECs and HMVECs, were purchased from Cambrex, Inc. (East Rutherford, NJ). The CD34+/AC133+ progenitors cells ($1–2 \times 10^5$ cells/ml) were grown in IMDM medium (Cambrex, Inc.) supplemented with 15% FBS (Invitrogen Corp., Carlsbad, CA), 50 ng/ml $VEGF_{165}$ (R&D Systems, Minneapolis, MN), 50 ng/ml rhbFGF (R&D Systems), and 5 units/ml heparin (Sigma Chemical Co., St. Louis, MO) on fibronectin-coated flasks (BD Biosciences, Franklin Lakes, NJ) at 37°C with humidified 95% air/5% CO_2 to generate EPCs (19, 29–31). Fresh media were added to the cultures every 3–5 days. The adherent cells that were generated from the original population of mixed nonadherent and adherent cells were designated EPC. The EPC were grown to confluency and could be passaged up to a dozen times. After the second passage, the EPC were maintained in IMDM media supplemented with 15% FBS without additional growth factors. The EPC were divided 2–3-fold at each passage. HUVEC and HMVEC were maintained in endothelial cell growth medium containing 2% FBS (EGM-2; Cambrex, Inc.) at 37°C with humidified 95% air/5% CO_2. Both of the donors for the AC133+/CD34+ progenitor bone marrow cells were normal male healthy volunteers. Both were Caucasian, ages 18 (donor 1) and 23 (donor 2), and both tested negative for HIV and hepatitis B and C infection.

The SKOV-3 human ovarian carcinoma cell line was purchased from American Type Culture Collection (Manassas, VA). SKOV-3 cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS.

Flow Cytometry. EPC, HMVEC, and HUVEC were collected by brief exposure to 0.25% tryspin/1 mM EDTA (Invitrogen Corp.) and washed twice in ice cold phosphate buffered 0.9% saline containing 5 mM EDTA and 5% FBS (FACS buffer). Approximately 2×10 (5) cells were suspended in a final volume of 100 μl of FACS buffer and incubated with a primary antibody for 1 h on ice. The cells were then washed twice with FACS buffer and incubated with secondary antibody, when necessary, for 45 min on ice. The cells were again washed twice with FACS buffer and suspended in a final volume of 500 μl for flow cytometric analysis.

The following primary antibodies were used at a 1:20 dilution: (*a*) anti-CD31-FITC (PharMingen, San Diego, CA); (*b*) anti-CD34-FITC (PharMingen); (*c*) anti-CD36-FITC (PharMingen); (*d*) anti-AC133-PE (Miltneyi Biotech, University Park, PA); (*e*) anti-CD105 (PharMingen); (*f*) anti-P1H12 (Chemicon International, Temecula, CA); (*g*) anti-CD54 (PharMingen); and (*h*) anti-CD106 (PharMingen). The following unconjugated primary antibodies were used at a 1:10 dilution: (*a*) anti-VEGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA) and (*b*) anti-VE-cadherin (Santa Cruz Biotechnology). Unconjugated primary antibody against CD36 (PharMingen) was used at a 1:100 dilution, and antibody against CD141 (PharMingen) was used at a 1:500 dilution. The following secondary antibodies were used at a 1:35 dilution: (*a*) antimouse-FITC (PharMingen or Jackson Immunoresearch, Bar Harbor, ME); (*b*) anti-rabbit-FITC (Santa Cruz Biotechnology); (*c*) anti-goat-FITC (Santa Cruz Biotechnology) or at a 1:50 dilution; (*d*) anti-mouse-PE (Jackson Immunoresearch). EPC and HUVEC were stimulated with 20 ng/ml TNF α (R&D Systems) for 48 h before CD106 staining. Cells were fixed in 4% paraformaldehyde and analyzed within 24 h. Positive expression was determined if cells gated at $\geq$10%.

Statistical Comparison of SAGE Libraries. SAGE libraries for brain, breast and colon tumor and normal endothelial cells (EC), and SAGE libraries from EPC and HMVEC were constructed as described previously (32). SAGE libraries for brain, breast EC, and EPC were constructed using the long SAGE technology (33). SAGE libraries for colon EC and HMVEC were constructed using microSAGE technology (34). The sample information for all of the libraries constructed is: three brain tumors and two normal brain samples, two primary breast tumors, one breast bone metastasis and one normal breast sample, one colon tumor and one normal colon sample, EPC grown with or without VEGF, and HMVEC in the presence or absence of DMSO. SAGE tags were normalized to 50,000 total library counts except the colon EC libraries, which were normalized to 100,000 total library counts. Long SAGE tags were converted to regular SAGE tags, and tag counts for the same regular SAGE tags were aggregated. There were 139,838 unique SAGE tags from the 15 libraries. SAGE tag counts of two or less were filtered out in $\geq$10 of the 15 libraries to remove erroneous tags. Within tissue comparison of tumor *versus* normal libraries were also performed through a χ^2 analysis on the averages of the normal and tumor SAGE tag counts. Confidence interval levels of 90, 95, and 99% were also used for tag filtering and generated 4030 and 762 tags, respectively. Hierarchical clustering and Venn diagrams were performed on filtered libraries using GeneSpring software release 5.0.2 build number 954 (Silicon Genetics, Redwood City, CA). Pearson correlation was for similarity measurement, and the minimum distance was set to 0.001.

Proliferation. The generation times of the EPC, HUVEC, and HMVEC were determined over a 4-day period. Cells were plated in 96-well plate format at 2000 or 3000 cells/well in triplicate. EPC were grown in EGM-2 with 2% FBS or IMDM with 15% FBS and HMVEC and HUVEC were grown in EGM-2 media with 2% FBS. Cells were assayed daily using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) that measures ATP levels. The results are expressed as cells/well +/− SD.

Migration Assay and Invasion Assay. EPC, HUVEC, or HMVEC (5×10^4 cells) were placed into the top chamber of a BD Falcon HTS FluoroBlok insert with a PET membrane with eight μm pores (BD Biosciences) in 300 μl of serum-free IMDM for EPC, EGM-2 for HUVEC, or HMVEC in triplicate. For the invasion assay, the FluoroBlok inserts were coated with a thin layer of Matrigel. The inserts were placed into the bottom chamber wells of a 24-well plate containing IMDM or EGM-2 media and FBS (0, 0.5, or 5%) as chemoattractant. For direct comparison of cell lines, 5% FBS was used. At 4, 24, and 48 h, cells that migrated through the pores of the membrane to the bottom chamber were stained with calcein 8 μg/ml (Molecular Probes, Eugene, OR) in PBS for 30 min at 37°C. The fluorescence of migrated cells was quantified using a fluorimeter set at 485 nm excitation and 530 nm emission. Data are expressed as number of cells that migrated through or invaded pores +/− SD.

***In Vitro* Tube Formation.** Matrigel (BD Biosciences) was added to the wells of a 48-well plate in a volume of 150 μl and allowed to solidify at 37°C for 30 min. After the Matrigel solidified, EPC, HUVEC, or HMVEC ($2–2.5 \times 10^3$ cells) were added in 300 μl of media: IMDM with 2% FBS for EPC and AC133+/CD34+ bone marrow progenitor cells, EGM-2 for HMVEC and HUVEC. The cells were incubated at 37°C with humidified 95% air/5% CO_2 for 24 h (35). The tube networks were stained with calcein and quantified by image analysis using Scion image as fluorescent pixel area.

Invasion Assay in Presence of Cancer Cell Clusters. Briefly, a thick layer of Matrigel (BD Biosciences) was added to the wells of a 24-well plate in a volume of 300 μl and allowed to solidify at 37°C for 30 min (36). A plug of Matrigel of ~1 mm diameter was removed using a glass pipette under light vacuum. The hole was filled with SKOV3 cells suspended in 1% collagen I (Cohesion Technologies, Palo Alto, CA) at a concentration of 1×10^6 cells in 5 μl and allowed to solidify at 37°C for 30 min. EPC or HMVEC were labeled with PKH67 green dye according to the manufacturer's instructions (Sigma). The cells were incubated in the presence of 2.5 μM dye suspended in diluent for 5 min. The labeling was stopped with 1 ml of FBS for 1 min followed by three washes in serum-containing medium. After the washes, the cells were suspended in IMDM or EGM-2 and counted by hemocytometer. EPC or

[2] The abbreviations used are: HMVEC, human microvascular endothelial cells; EPC, endothelial precursor cells; VEGF, vascular endothelial growth factor; ICAM, intercellular adhesion molecule; HUVEC, human umbilical vein endothelial cell; SAGE, serial analysis of gene expression; FBS, fetal bovine serum; $VEGF_{165}$, vascular endothelial growth factor; rhbFGF human recombinant basic fibroblast growth factor; FACS, fluorescence activated flow cytometry; bFGF, basic fibroblast growth factor; DAPI, 4, 6-diamidino-2-phenylindole.

HMVEC (3×10^5 cells) were added to each well in 300 μl of IMDM or EGM-2 media. The cultures were incubated at 37°C in humidified 95% air/5% CO_2 for 24 or 48 h. The EPC or HMVEC in the wells were visualized using a fluorescein (PKH67) filter on an inverted phase using a fluorescent inverted phase microscope.

***In Vivo* Matrigel Angiogenesis Assay.** EPC were prelabeled with DAPI (Sigma) at 20 μg/ml at 37°C for 20 min, then were washed twice with PBS and used within 24 h. The EPC were collected by exposure to 0.25% trypsin. Approximately 5×10^5 EPC in 100 μl of PBS were mixed with 500 μl of Matrigel (BD Biosciences) containing 40 units/ml heparin and 150 ng/ml rhbFGF. The Matrigel containing EPC mixtures (500 μl) were implanted s.c. into the mid-dorsal region of female nude mice. The Matrigel plugs containing EPC were collected after 7 days *in vivo* and snap frozen in OCT medium. For detection of DAPI-labeled EPC, 5-μm frozen sections of the Matrigel plugs were rinsed briefly with PBS, fixed in 10% formalin for 10 min, washed twice, and then imaged by fluorescent microscopy after mounting. Other sections were stained with H&E and imaged by bright field microscopy or stained with mouse antihuman CD31 (1 μg/200 μl/slide; clone JC70A; DAKO, Carpinteria, CA) or rabbit antihuman von Willebrand factor (DAKO) using a Cy3 secondary antibody for immunohistochemistry.

RESULTS

In the absence of stimulation, AC133+/CD34+ bone marrow cells can be maintained for a short period of time in culture with little expansion potential. On exposure to the angiogenic factors $VEGF_{165}$, rhbFGF, and heparin, the AC133+/CD34+ progenitor cells began to proliferate. Within 1–2 weeks, a new phenotype of cells began to emerge and adhere to the flask. After 2 weeks, a confluent, adherent monolayer of elongated cells was obtained. These adherent cells derived by angiogenic growth factor exposure of AC133+/CD34+ progenitor cells in culture were designated EPC. The remaining bone marrow cells in suspension that continued to proliferate and thrive were transferred to a new flask for additional exposure with the angiogenic factors $VEGF_{165}$, rhbFGF, and heparin and generation of EPC. The EPC maintain significant expansion potential and can be passaged at least ≤12 times. For the experiments described herein, the EPC were limited to 10 passages. Maintenance of the EPC at a minimum of 50–60% confluence was important to generating high cell numbers with a doubling time of ~3 days. EPC viability remained highest when at near confluence in culture.

The expression of cell surface proteins that are characteristically expressed on bone marrow progenitor cells and mature ECs was assessed on EPC in the presence and absence of EC-associated growth factors and on HUVEC and HMVEC (Table 1). Flow cytometry was used to score relative expression of each marker on the various cell types. AC133/CD133 is a M_r 97,000 five-span transmembrane protein with no known function (37, 38). The expression of the AC133 protein is, in large part, limited to normal bone marrow and some CD34+ leukemias. The expression of progenitor cell marker AC133 on the cell surface EPC was weak. However, AC133 was not detectable on the surface of the mature ECs represented by HUVEC and HMVEC. Sialomucin/CD34 was also a marker in the bone marrow progenitor cell population selected to be the originating cell for EPC development. CD34 is found expressed in vessels *in vivo* and on ~20% of HUVEC and HMVEC in cell culture (39). EPC, HUVEC, and HMVEC in the current study were weakly positive for CD34 expression.

Several cell surface EC markers were similarly expressed on the EPC, HUVEC, and HMVEC. Among the similarly expressed markers were VEGFR2/Flk-1, endoglin/CD105, and P1H12/CD146. VE-cadherin and gPIIIB/CD36 were expressed weakly in EPC, HUVEC, and HMVEC. Several markers differentiated EPC from mature ECs represented by HUVEC and HMVEC. ICAM1/CD54, ICAM2/CD102, and thrombomodulin/CD141 were much more strongly expressed on the mature ECs than on the EPC. Finally, PCAM/CD31 was very strongly expressed on the mature ECs represented by HUVEC and HMVEC and was more weakly expressed on the surface of EPC.

In other studies, EPC were tested for uptake of acetylated LDL and binding to UEA-1 lectin, traits that are common for mature ECs, such as HUVEC and HMVEC. The cells were incubated for 4 h at 37°C with 10 μg/ml Dil-Ac-LDL (Biomedical Technologies, Stoughton, MA) or with FITC-labeled UEA-1 lectin (Sigma) for 1 h at 37°C in serum-free media. All cells were washed twice with PBS after incubation. Although HMVEC and HUVEC demonstrated robust uptake of AcLDL and binding of UEA-1 lectin, the EPC did not take up AcLDL and weakly bound UEA-1 lectin (data not shown). The differences between cell surface markers expressed by the EPC and the HUVEC and HMVEC suggest that the EPC population derived in cell culture does not express all of the characteristic markers associated with fully mature ECs. Thus, EPC may be regarded as representing an intermediary cell type between the AC133+/CD34+ progenitor cell and the mature well-differentiated EC.

Gene expression in the EPC was compared with gene expression from human tumor ECs and with gene expression in HMVEC using SAGE analysis (Refs. 33 and 34; Fig. 1). Human tumor ECs were derived from three breast tumors, three brain tumors, and one colon tumor. The seven tumor EC SAGE libraries were compared with the corresponding normal tissue EC SAGE libraries, and the gene expressed at significantly higher levels in the tumor ECs were determined by χ^2 analysis. The genes expressed in the EPC and HMVEC as determined by SAGE analysis were compared with the genes expressed at three levels of stringency in the pooled tumor EC libraries. At each level of stringency, ≥99%, ≥95%, and ≥90%, there were a larger number of expressed genes in common between the EPC and the tumor ECs than between the HMVEC and the tumor cells. Thus, at the gene expression level, there was greater similarity between EPC and tumor ECs than between HMVEC and tumor ECs.

The performance of EPC in several cell-based assays in comparison with the mature ECs commonly used in the angiogenesis field, HUVEC and HMVEC, was assessed. EPC were evaluated in proliferation, migration, invasion through Matrigel, and tube/network formation assays. These assays are routinely used to identify and evaluate both proangiogenic and antiangiogenic agents that may be potentially effective in therapeutic clinical settings. Generation times

Table 1 *Comparison of cell surface molecular marker protein expression by EPC, HUVEC, and HMVEC*

The cells were immunostained for the markers and analyzed by flow cytometry. Relative expression of % gated or positive cells are shown: 1 = 0–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100%. EPC grown in the presence or absence of growth factors (GF; $VEGF_{165}$, rhbFGF, and heparin) were compared.

	Molecular marker											
Cell type	AC133 CD133	Sialoumucin CD34	P1H12 CD146	VEGFR2 Flk1	PCAM CD31	Endoglin CD105	VE-cadherin	ICAM1 CD54	VCAM1 CD106	ICAM2 CD102	gPIIIB CD36	Thrombomodulin CD141
EPC + GF	1	1	3	3	2	4	1	1	0	0	1	0
EPC	1	1	4	3	3	4	1	2	2	1	1	1
HUVEC	1	1	4	3	4	4	1	4	4	4	1	4
HMVEC	1	1	4	3	3	4	1	4	2	4	1	4

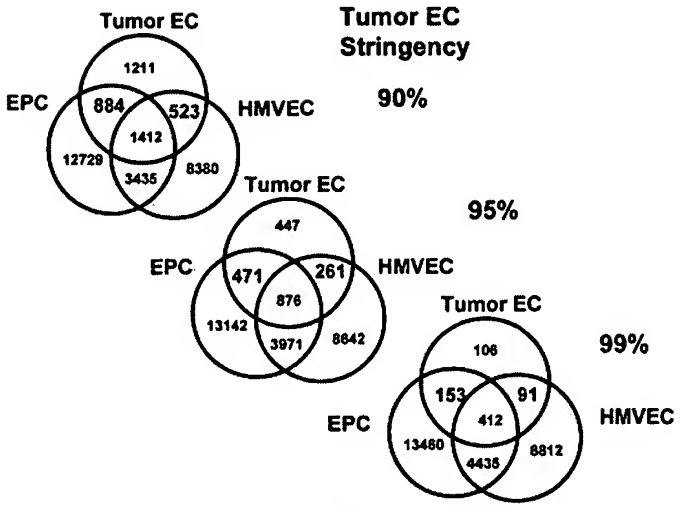

Fig. 1. Schematic showing overlaps in gene expression determined by SAGE analysis for tumor ECs derived from human surgical specimens of three breast tumors, three brain tumors, and one colon tumor and EPC and HMVEC grown in cell culture. The SAGE gene expression data from the seven tumor EC libraries were pooled, and genes were expressed at higher levels in the tumor endothelium compared with normal ECs derived from one normal breast, two normal brain, and one normal colon specimen at ≥99%, ≥95%, and ≥90% confidence levels by χ^2 analysis.

were determined for HMVEC, HUVEC, and EPC over 96 h (Fig. 2). EPC were grown in either IMDM plus 15% FBS or in complete EGM-2 media that is supplemented with VEGF, bFGF, and 5% FBS. The generation times for EPC in IMDM media with high serum were ~117 h, similar to HMVEC with a generation time of 115 h. When EPCs are grown in EGM-2 media, the generation time decreased to ~36 h, a rate more similar to HUVEC with a doubling time of ~27 h. These results indicate that like mature ECs, EPC respond to growth factors, such as VEGF and bFGF. EPC proliferation rates resemble both HMVEC and HUVEC and comparisons are dependent on the media chosen.

CD34+/VEGFR2+ cells from peripheral blood have been reported to migrate in response to VEGF and bFGF, leading to further differentiation and maturation of a subset of those progenitor cells that were AC133+ (21). The EPC generated in cell culture from AC133+/CD34+ bone marrow progenitor cells exposed to $VEGF_{165}$ and rh-bFGF was investigated in a migration assay designed to evaluate their chemotactic capacity. The cells in serum-free media were placed in an insert with a membrane containing 8-μm pores. The insert was placed in a well containing media with FBS at increasing concentrations as chemoattractant. Fig. 3*A* depicts the time course of EPC migration at 4, 24, and 48 h in the presence of 0, 0.5, and 5% FBS. EPC began to migrate within 4 h in a FBS concentration-dependent manner and continue to migrate up to 48 h by which time there is likely no longer a serum gradient between the insert and well. Because EPC do not proliferate significantly in a low FBS media, the increase in fluorescence over 48 h indicates a continued migration by the EPC through the pores of the insert membrane. Although the presence of FBS as chemoattractant was observed at the earliest time point, by 24 and 48 h, migration of EPC proceeded as well in the absence of FBS as in the presence of FBS. Thus, the EPC can migrate even in the absence of serum (Fig. 3*A*).

When 0.5% FBS was used as the chemoattractant at 24 and 48 h, the number of cells migrating was very similar when EPCs were compared with HUVEC and HMVEC (Fig. 3*B*). After the EPC population of cells was established by passing twice in the presence of the endothelial growth factors, $VEGF_{165}$, rhbFGF, and heparin, the EPC were maintained without addition of growth factors to the media. To determine whether continuous stimulation with $VEGF_{165}$, rhbFGF, and heparin would affect the EPC behavior in culture, some EPC were maintained in growth factor-rich media for an additional three cell passages. As can be seen in Fig. 3*B*, there was no difference in the ability to migrate between EPC maintained without growth factors and those maintained in growth factor-rich media.

Invasion through Matrigel is another important property recognized as a characteristic of ECs. The invasion assay used the same insert and well apparatus as the migration assay except that a layer of Matrigel coating the porous membrane through which the cells invade before they can migrate through the pores of the membrane. The invasion by EPC, HUVEC, and HMVEC was examined at 24 and 48 h with 0.5% FBS as the chemoattractant (Fig. 4). The EPCs performed as well as the mature ECs, HUVEC and HMVEC, in the cell culture Matrigel invasion assay. In addition, AC133+/CD34+ bone marrow progenitor cells from a second donor were differentiated in EPC. The EPC generated from both individual donors performed equally well in the cell culture Matrigel invasion assay.

Angiogenesis, a critical step in tumor development, is hypothesized to occur through the secretion of angiogenic growth factors and cytokines by malignant cells and perhaps other normal cells in the vicinity of the emerging tumor. The gradient of angiogenic factors attracts EPC in a process resembling vasculogenesis and activates sprouting from existing endothelium causing neovascularization. The coculture assay including human SKOV3 ovarian cancer cells suspended in a collagen plug surrounded by Matrigel allows assessment

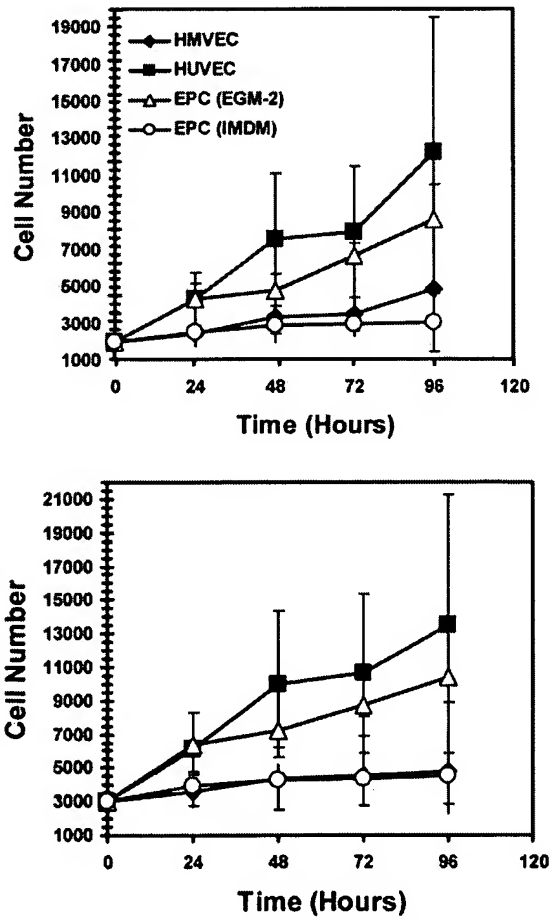

Fig. 2. Growth curves for EPC, HUVEC, and HMVEC under optimal culture conditions over the course of 96 h. *Left panel*, cellular proliferation beginning with 2000 cells/well. *Right panel*, cellular proliferation beginning with 3000 cells/well. The data are the mean ± SD for three independent experiments.

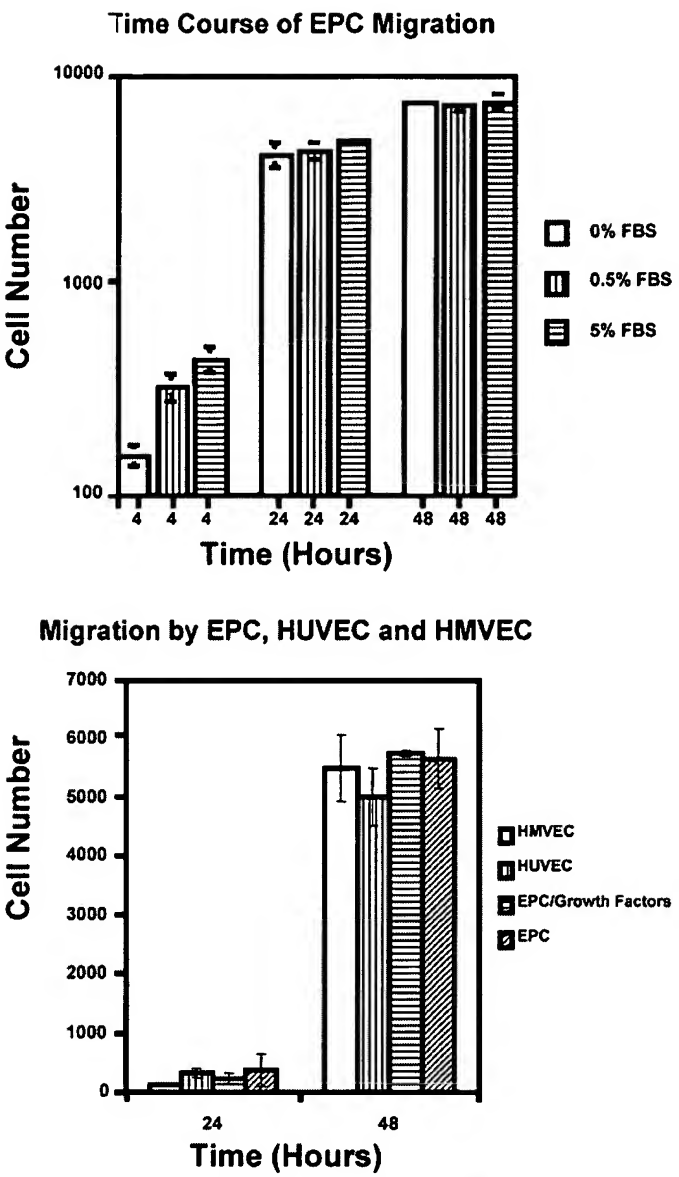

Fig. 3. *Left panel,* time course of EPC migration in response to various concentrations of FBS. The data are the mean ± SE for three independent experiments. Migration toward a serum stimulus is significantly greater at 4 h with $P < 0.001$, and migration toward 5% FBS is significantly greater than migration in the absence of serum stimulus at 24 h with $P < 0.05$. *Right panel,* comparison between EPC, HUVEC, and HMVEC in the migration assay. The effect of $VEGF_{165}$, rhbFGF, and heparin on the migration of EPC was also investigated. The data are the mean ± SD for three independent experiments.

of the invasion potential of cells under the influence of factors secreted by the malignant SKOV3 tumor cells (Fig. 5). EPC and HMVEC were evaluated for their ability to respond to human SKOV3 ovarian cancer cells in the three-dimensional coculture assay. The SKOV3 cancer cells (1×10^6 cells) were clustered within a 1 mm^3 collagen plug surrounded by Matrigel in a 24-well plate. EPC or HMVEC (3×10^5 cells) fluorescently labeled with PKH67 were added to the well, and their mobility was monitored for 48 h. As shown in Fig. 5, EPC but not HMVEC invaded into the SKOV3 ovarian cancer cell clusters and concentrated into the center of the clusters. Thus, EPC can be differentiated from mature ECs represented by HMVEC by their ability to invade through Matrigel and collagen under the influence of factors secreted by human SKOV3 ovarian carcinoma cells.

The ability to form tubes or networks in Matrigel is a hallmark of EC behavior that models the formation of new vessels or vasculature *in vivo*. For the tube/network formation assay, AC133+/CD34+ bone marrow progenitor cells, EPC, HUVEC, and HMVEC, were plated onto a layer of Matrigel and allowed to incubate for 24 h (Fig. 6). The more undifferentiated AC133+/CD34+ bone marrow progenitor cells did not form tubes or networks on the Matrigel. However, the EPC formed tubes/networks that appear quite similar to those formed by HUVEC and HMVEC. Thus, differentiation of the $CD34^+$/$AC133^+$ bone marrow progenitor cells toward the EC phenotype as represented by EPC allows the cells to form tubes/networks on Matrigel indicating that on exposure to proangiogenic factors, cells derived from bone marrow can develop several properties similar to mature ECs, like HUVEC and HMVEC.

To develop a convenient *in vivo* model for testing potential anti-angiogenic agents against human vascular targets expressed on EPC, EPC (5×10^5 cells) labeled with a tracer amount of the fluorescent nuclear-stain DAPI were mixed into Matrigel (500 μl) and injected s.c. into nude mice. After 7 days, the cell-laden Matrigel plugs were collected and snap frozen. Sections from the plugs were evaluated for tube/network formation and retention of the EPC (Fig. 7, *A–E*). The tubes/network formed throughout the plugs, and apparent degradation of the Matrigel support was visualized by staining with H&E (Fig. 7, *A* and *B*). Fluorescence microscopy allowed visualization of the nuclei of DAPI-labeled EPC in the tubes/network (Fig. 7*C*). Fig. 7*D* shows staining of the EPC for CD31, and Fig. 7*E* shows staining for von Willebrand's factor by fluorescent immunohistochemistry.

DISCUSSION

Research has shown that progenitor cells derived from adult human bone marrow or from umbilical cord blood and can be recruited into circulation and give rise to well-differentiated cell types. VEGF and bFGF in particular have been implicated in the differentiation of these circulating progenitor cells into ECs (29). VEGF has been shown to modulate postnatal EPC kinetics in normal mice by increasing migration and chemotaxis (15, 16). Stromal cell derived factor-1 and other cytokines up-regulate MMP-9, which is required in the recruitment of hematopoietic stem cells and EPC from bone marrow (17). Insulin-like growth factor-1, granulocyte colony-stimulating factor, and stem cell growth factor also can drive progenitor cells toward an endothe-

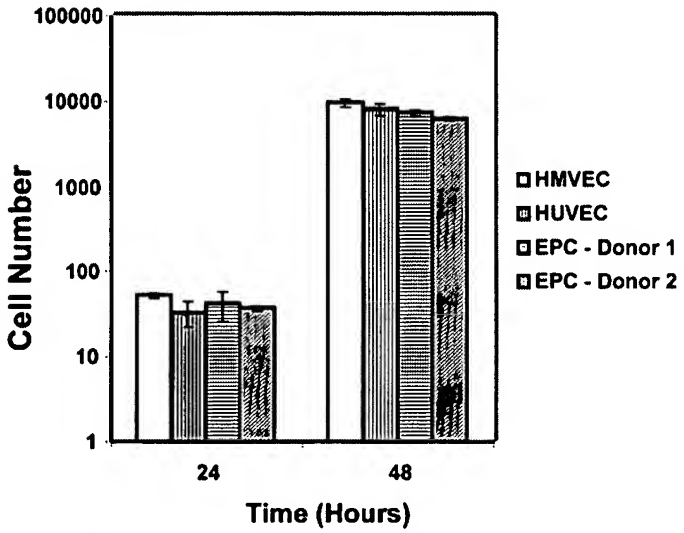

Fig. 4. EPC, HUVEC, and HMVEC were evaluated for their ability to invade through a layer of Matrigel. EPC derived from more than one donor showed similar results. Invasion by HMVEC is greater than the invasion by EPC from donor 2 at 24 h with $P < 0.01$ and at 48 h with $P < 0.05$. The data are the mean ± SD for three independent experiments.

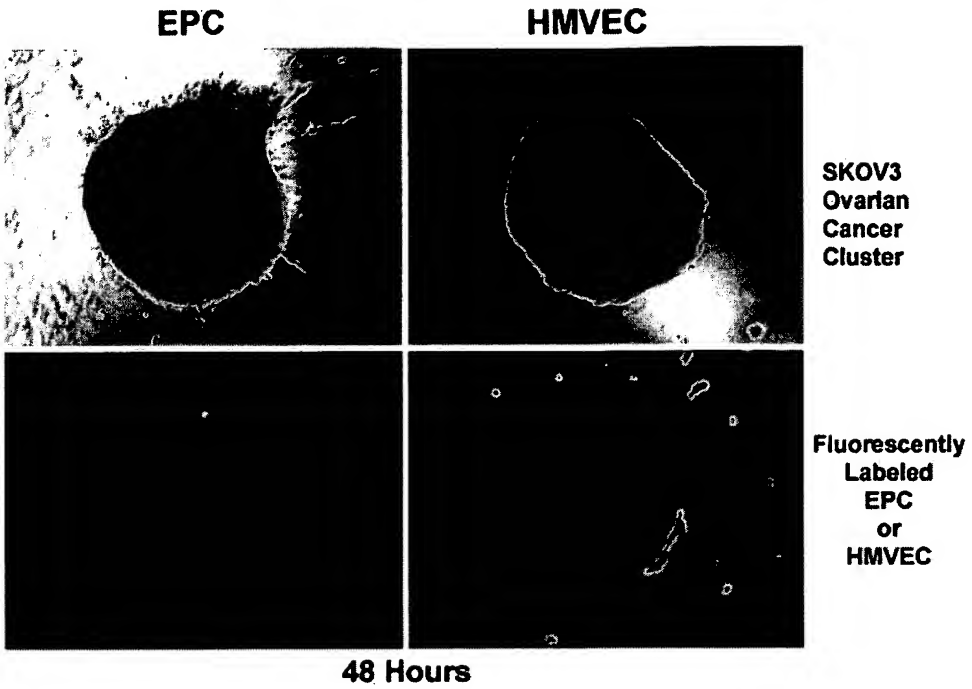

Fig. 5. SKOV3 human ovarian cancer cells are imbedded in a collagen plug surrounded by Matrigel on which were plated EPC or HMVEC labeled with green fluorescent PKH67 in a 24-well plate (36). Fluorescence was used to locate the EPC or HMVEC in the cultures after 48 h. Fluorescence inside SKOV-3 cancer cell clusters indicate invasion by EPC, whereas that lack of fluorescence in the HMVEC-containing wells indicate inability of the HMVEC to invade.

lial phenotype. Thus, EPC maturation can occur under a multitude of conditions supporting the notion that the multifaceted potential these progenitor cells possess enables them to function and respond to different pathological settings.

ECs can arise from a subset of common hematopoietic stem cell precursors identified by the markers AC133 and CD34. In cell culture on exposure to $VEGF_{165}$, rhbFGF, and heparin, the loss of AC133 expression occurs early as the progenitor cells differentiate through stages to a cell with a phenotype resembling ECs, herein described as EPC. The EPC generated express several molecular markers associated with ECs, such as P1H12, VEGFR2, PECAM, and endoglin, and demonstrate migration properties very similar to HUVEC and HMVEC (30, 31, 39). However, other EC markers, such as thrombomodulin, ICAM1, ICAM2, and VCAM1, were not found on EPC. The adhesion molecules ICAM and VCAM mediate the interaction between ECs and T and NK cells, as well as between ECs and stromal tissue or cancer cells (40). Thrombomodulin is a cell surface anticoagulant that modulates the activity of the hemostatic protease thrombin and blocks thrombin's procoagulant effects and enhances thrombin-dependent activation of anticoagulant protein C (41, 42).

The EPC were obtained from bone marrow cells expressing CD34 and AC133; however, the full potential of this subpopulation of progenitor cells remains to be elucidated. Although expression of AC133 protein appears to be limited to bone marrow and some leukemias from immunohistochemical staining, the message for AC133 is present in other tissues, including kidney and pancreas (37). It is possible that under specific stimulatory conditions that AC133+ progenitor cells can differentiate into various cell types. A second isoform of AC133 expressed in human stem cells other than hematopoietic tissue has been identified (38).

The EPC examined in these studies are likely intermediary between early progenitor cells and fully mature ECs. Like HUVEC and HMVEC, EPC have the capacity to migrate, invade through Matrigel, and form tubes/networks on a Matrigel-coated substrate. The *in vivo* environment cannot be wholly mimicked in culture, and all of the components that contribute to the maturation and maintenance of ECs

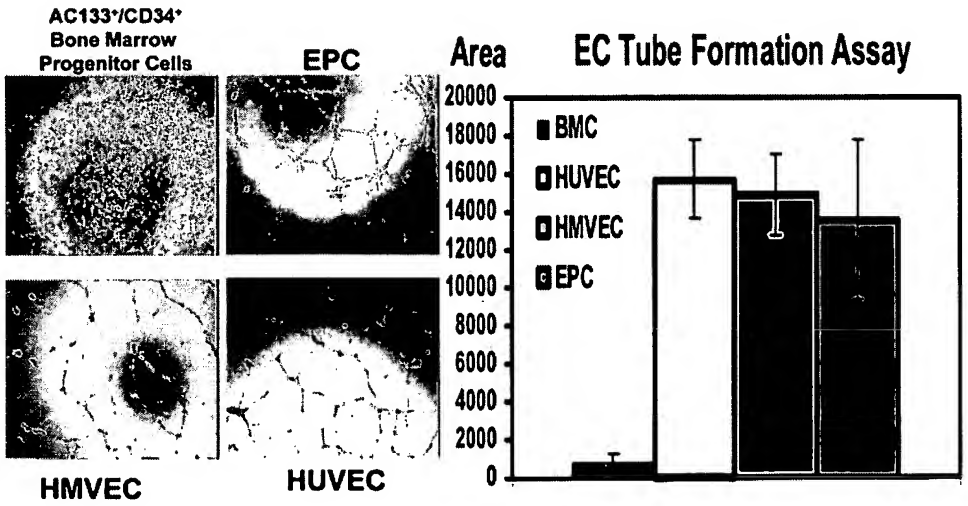

Fig. 6. The ability of EPC, AC133+/CD34+ bone marrow progenitor cells, HUVEC, and HMVEC to form tubes/networks on Matrigel over 24 h was compared. The tubes were visualized using calcein staining and Scion image analysis to derived pixel area. The data are the mean ± SD for three independent experiments.

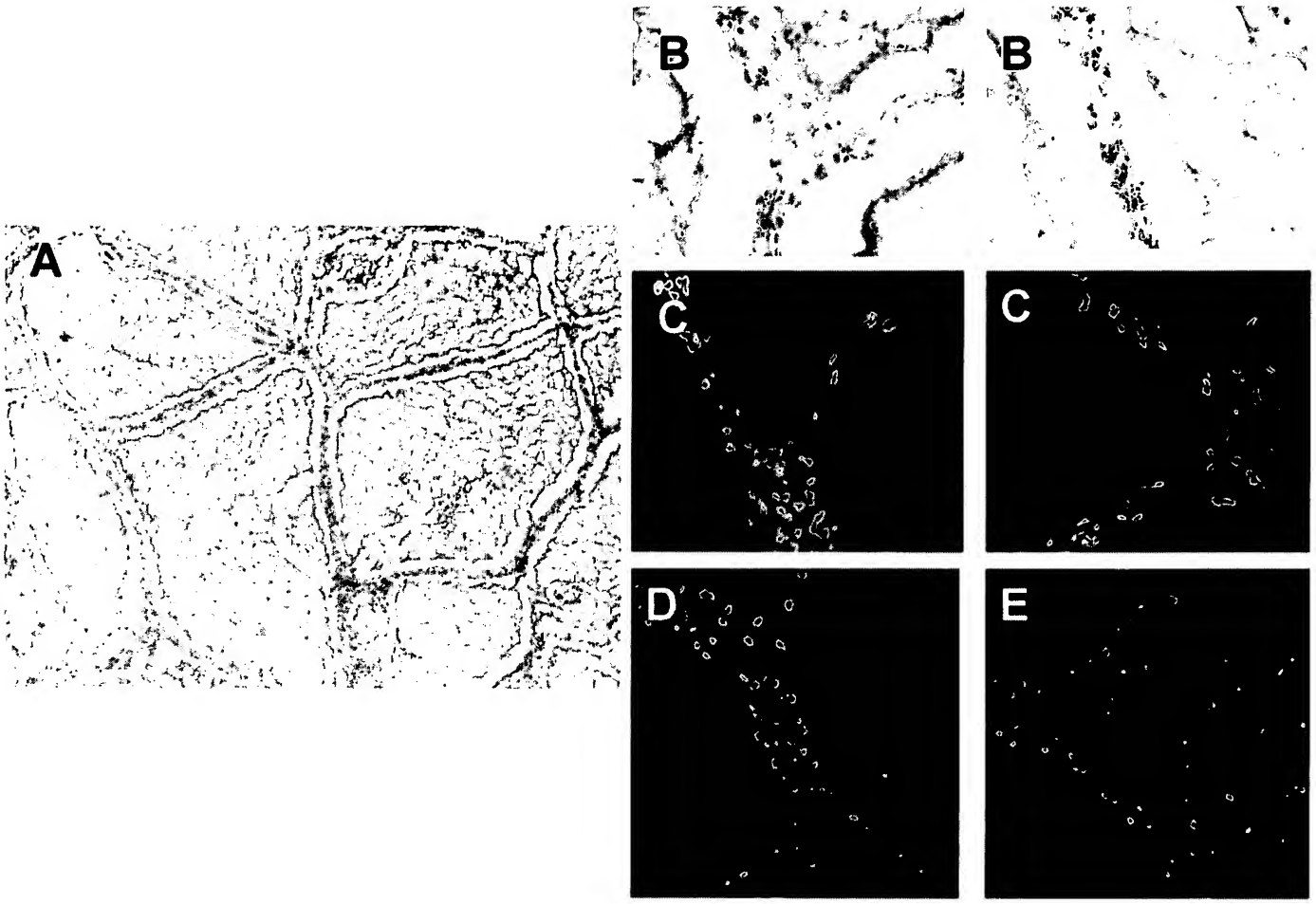

Fig. 7. EPC prelabeled with DAPI was mixed with Matrigel and injected s.c. into nude mice in a 500-μl volume to form a plug. *A*, image of an H&E-stained section of an EPC-containing Matrigel plug at ×20 magnification shows tube/network formation after 7 days *in vivo*. *B*, image of an H&E-stained section of an EPC-containing Matrigel plug at ×40 magnification. The pink web-like pattern is the remaining Matrigel. *C*, fluorescence image of DAPI-stained EPC in a Matrigel plug at ×40 magnification. *D*, fluorescence image of human CD31 staining in *red* (Cy3) and DAPI staining of all nuclei in *blue*. *E*, fluorescence image of human von Willebrand factor (Factor VIII) staining in *red* (Cy3) and DAPI staining of all nuclei in *blue*.

have yet to be fully characterized. However, there was a clear difference in the behavior of EPC and HMVEC in the coculture assay where human SKOV3 ovarian cancer cells provided the stimulus for vasculogenesis/neoangiogenesis. In that assay, the EPC were able to invade into the tight cluster of malignant cells, whereas the HMVEC did not have the capacity for invasion. SAGE analysis for gene expression allowed us to compare EPC and HMVEC to gene expression in tumor ECs isolated from clinical surgical samples of breast, colon, and brain cancer. The data show that EPC are more similar in expressed genes to tumor EC than are HMVEC. Loading human EPC into Matrigel and the injection of the Matrigel as a s.c. plug into murine hosts resulted in formation of a network/vasculature that was likely a mosaic of human and mouse cells after 7 days. On visualization of the DAPI-labeled EPC, it was evident from the presence of unlabeled cells that some regions of the vasculature that were not comprised of EPC but rather consisted of murine ECs. Another possibility is that host macrophages could enter the Matrigel and engulf the human EPC; however, the number of pyknotic cells in the Matrigel plugs was very low (0.1%), and macrophage-like cells were seen. The anastomses of the EPC and host ECs have generated a basic model of human vasculature in a murine host. Preclinical models comprised at least in part of ECs of human origin are valuable in evaluating the efficacy of potential antiangiogenic therapeutics. This model is, however, simpler to execute than bone marrow transplant or skin xenograft models (43–46).

Endothelial progenitor cells derived from the bone marrow can be found in circulation in adults and may be recruited to and incorporated into neovascularization at sites of wounds and tumor vascularization (10, 15, 16). Endothelial progenitor cells isolated from the circulation have a relatively high proliferation rate compared with mature ECs shed from the blood vessel wall (18). In the cancer patient populations, circulating endothelial progenitor cells and EPC may be used as a surrogate marker/biomarker of response to an antiangiogenic or cytotoxic therapy. Endothelial progenitor cells and EPC have been identified in circulation and in the vicinity of the malignant cells in patients with multiple myeloma, astrocytoma, and inflammatory breast cancer, and additional malignancies will no doubt be identified (47–49). Shirakawa *et al.* (47) found a significantly higher population of tumor-infiltrating ECs or EPC in tumor-associated stroma of inflammatory breast cancer specimens than in noninflammatory breast cancer specimens using immunohistochemical staining for Tie2, VEGF, Flt-1, and CD31. There is potential value for EPC outside the field of oncology for treating vascular diseases by engraftment or as drug delivery systems. Continued characterization of EPC, effects of cytokines and growth factors on EPC differentiation, and identification of the capabilities of EPC in preclinical and clinical settings will continue is important.

Like other areas of drug discovery, the field of antineoplastic antiangiogenic drug discovery has been hampered by the use of nonideal models for human tumor vasculature and endothelium. The

cell-based models that have been the standard for the field, HUVEC and HMVEC, are mature, well-differentiated, normal human ECs. Through the study of genes expressed in human tumor ECs isolated from fresh surgical specimens of human tumors and corresponding normal tissues as determined by SAGE analysis, it is clear that human tumor endothelium is not well represented by HUVEC and HMVEC. The search for better cell-based models for human tumor ECs has yielded the EPC which in cell culture were developed from AC133+/CD34+ bone marrow progenitor cells. The EPC retain the basic functions of migration and tube formation and have greater proliferative capacity and greater invasive capacity than HUVEC and HMVEC. Recently, endostatin has been shown to inhibit the mobilization of murine EPC in tumor-bearing mice into circulation and reduce the effectiveness of xenotransplantation of human bone marrow cells into SCID mice (27). Utilization of EPC rather than HUVEC or HMVEC in drug discovery for evaluating potential antiangiogenic therapies in the preclinical setting may result in the selection of targets and agents that will prove to be more effective in the clinic.

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ANGIOGENESIS INHIBITION: A REVIEW

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Abstract—In this review we discuss the concept of anti-angiogenesis, which is the inhibition of neovascularization. Anti-angiogenic agents are viewed from the standpoint of their effect on various elements of the angiogenic process, including induction of vascular discontinuity, endothelial cell movement, endothelial cell proliferation, and three-dimensional restructuring of patent vessels. An effort is made to place the many different approaches to anti-angiogenesis research into a comprehensible structure, in order to identify problems of evaluation and interpretation, thereby providing a clearer basis for determining promising and needed directions for further investigation.

Keywords—Neovascularization, anti-angiogenesis, endothelial cell proliferation, tumor growth, endothelial cell migration, blood vessel formation.

CONTENTS

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Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; CAM, chorioallantoic membrane; FGF, fibroblast growth factor; HUVE, human umbilical vein endothelial; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; PAF, platelet-activating factor; PAI, plasminogen activator inhibitor; PECAM-1, platelet–endothelial cell adhesion molecule-1; PF4, Platelet Factor 4; PMA, phorbol myristate acetate; SPARC, Secreted Protein, Acidic and Rich in Cysteines; TGF, transforming growth factor; TIMP, tissue inhibitors of metalloproteinases; TNF, tumor necrosis factor; TSP, thrombospondin; uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor.

1. INTRODUCTION

1.1. The Concept of Angiogenesis

The endothelium, a tissue comprised of a monolayer of cells lining the lumen of blood vessels, is the most quiescent of all tissues in the body. It is almost impossible to find any mitotic figures

in vascular endothelium under normal conditions, yet it can become one of the most rapidly proliferating of all cell types when stimulated. It is the sole kind of cell in capillaries, and it is in these hair-like vessels that endothelial cell proliferation occurs during the regeneration, repair and establishment of new blood vessels. The process leading to the formation of these blood vessels is called *angiogenesis* or *neovascularization.*

In an attempt to bring precision to the understanding of this process, investigators have used the term "*vasculogenesis*" to denote the development of new blood vessels from primitive precursor cells (angioblasts) during embryogenesis (Noden, 1989), reserving the term "angiogenesis" to the elaboration of blood vessels by endothelial cell outgrowth from preexisting vessels. The term *re-endothelialization* is also used where the normal blood supply by capillaries is being reestablished after trauma, as in the case of wound healing or in the repair of localized lesions.

Judah Folkman was the pioneer in the area of angiogenesis, writing extensively about tumor angiogenesis as early as the 1970s. He observed that tumors remain in a state of dormancy until they establish a blood supply, but once having induced vessels to provide nutrients and other factors, they grow with malignant rapidity. In the ensuing years, he and many others have shown that angiogenesis is a vital component of embryonic processes such as placentation, limb development or vascularization of the brain. Angiogenesis is a prominent feature of such normal body cyclical states as hair growth or menses. Angiogenesis is a significant element in a striking variety of disease states as well. *Neovascularization*, as it is more commonly called by ophthalmologists, is a feature of a long list of eye diseases, and in the case of diabetic retinopathy, is a major contributing cause of blindness. In dentistry, it accompanies gingivitis; in dermatology, it is a significant component of psoriasis, eczema and scleroderma. Immunologists recognize angiogenesis as an accompaniment of most, if not all, autoimmune diseases, as well as of inflammation. Surgeons rely on angiogenesis for successful repair of wounds; radiologists monitor the establishment of collateral blood vessels following myocardial infarcts.

1.2. The Process of Angiogenesis

Angiogenesis results from an ordered set of events, even though it may be evoked by a variety of angiogenesis-inducing agents.

Angiogenesis depends primarily, if not entirely, on endothelial cells, for, as stated, it is endothelial cells that line the lumen of all blood vessels and comprise the entirety of capillaries. Initiation of the process requires that endothelial cells, quiescent ($< 0.01\%$ in cell cycle) in their contact-inhibited state within an existing vessel, are freed from that inhibition. This may occur by mechanical disruption, as in wounding, or by dissolution of the basement membrane, which provides the architectural matrix for continuity of the vasculature. Once released from contact inhibition, endothelial cells leave the previously intact vessel by migrating in the direction of an angiogenic stimulus. Because endothelial cells do not exist singly (as, for example, leukocytes) but rather are organized as a tissue, migration must be supported by proliferation, so that, as endothelial cells move distally from the existing vessel, they preserve continuity by the proximal addition of new endothelial cells. Even as they move, the endothelial cells begin to organize into three-dimensional structures, and because of this continuity with the existing vasculature, manifest patency, so that only a few cells remain distal to blood-filled vascular sprouts. Thus, the angiogenic "cascade", as it has been referred to frequently, can be divided into four distinct phases: establishment of vascular discontinuity, cell migration, proliferation and structural reorganization.

A number of excellent reviews and symposium volumes provide fine overviews of the process of angiogenesis, as well as of the factors by which it is regulated (Gullino, 1985; Folkman, 1985b, 1991; Tseitlin, 1985; Folkman and Klagsbrun, 1987; Moses and Langer, 1991a; Ribatti *et al.*, 1991; Polverini *et al.*, 1991; Klagsbrun and D'Amore, 1991; Montesano, 1992; Bouck, 1993). A broader perspective on endothelial cell biology can be obtained from the various review chapters included in the volumes edited by Simionescu and Simionescu (1988, 1991) and Ryan (1989).

1.3. GENERAL CONSIDERATIONS

Our goal in this review is to organize the studies in the area of anti-angiogenesis from the standpoint of the process of angiogenesis: to attempt to identify the place or places in the sequence of events of the angiogenic process that are inhibited or abrogated, beginning with the disturbance of vascular continuity and ending with the completion of three-dimensional reorganization and the establishment of vessel patency and blood flow. There will be some anti-angiogenic agents for which the point of interference has not been identified as yet, and of necessity, the discussion of these will be limited to a general description without assignment to any particular phase of the angiogenic process.

We will assume that angiogenesis, however induced, is similar in terms of the angiogenic response, but there is no compelling evidence that this is so. For example, we do not really know whether angiogenesis in the developing embryo is similar to that which is induced by solid neoplasms or how it is achieved in different pathological situations.

We will also assume that all endothelial cells can participate in angiogenesis, but this assumption may not be correct. Are there, for example, a minority of primitive endothelial cell precursors that are the progenitors of new endothelial cells, or do all quiescent endothelial cells have the potential to proliferate and migrate in response to angiogenic stimulation? Is there a subset of cells that acquires responsivity to angiogenesis-inducing factors as a result of an initial stimulus, and do different subsets respond to different stimuli? We know for example that interferon (IFN)-γ can elicit a dramatic increase in several cell-surface associated molecules, including cell adhesion molecules and Class I and Class II major histocompatibility complex determinants, but that the response is not evoked in all endothelial cells. On the other hand, we do not know to what extent these early cell changes are required for subsequent neovascular responses.

Many disparate findings will be discussed during the course of this review. In large part, this will be due to the diverse assays that are used to characterize angiogenesis and to document inhibition. To some extent, this will be due to the fact that not all endothelial cells are alike and that they may respond differentially to various inducers or inhibitors of migration or proliferation. Furthermore, not all angiogenesis is alike, and there are significant differences between the vascular response to inflammatory mediators and the response to tumor-derived angiogenic factors. We will discuss conclusions from experimental results obtained with an impressive variety of assay systems (Auerbach *et al.*, 1991a), which may identify different components of the angiogenic response. We will try to put into context observations made *in vitro* with those made *in vivo*, and to synthesize information gained from normal processes in healthy individuals with those found to accompany such diverse diseases as psoriasis, retinopathy and various malignancies.

2. ANGIOGENESIS: ITS COMPONENT PARTS

2.1. INDUCTION OF VASCULAR DISCONTINUITY

2.1.1. *Proteolytic Enzymes*

Proteolytic enzymes secreted by tumor cells, released by macrophages or liberated following cell damage, are probably the most widespread stimulus for angiogenesis, their secretion leading to the disruption of basement membranes. A variety of metalloproteinases lead to breakdown of the collagens that provide the structural framework of the basement membrane. Both urokinase and tissue plasminogen activator generate active proteases that mediate matrix breakdown. Other proteases, released as the result of lysosome breakdown accompanying cytolysis, act on one or more of the constituents of the basement membrane.

2.1.2. *Local Cell Destruction*

Cell destruction (cytolysis) can be mediated by a profusion of agents ranging from systemically administered cytotoxic drugs to specific, locally acting cytotoxic T-cells. Whether cell death results from mercuric chloride, from the action of parasites, from oxygen excess and superoxide

production or from the release of porins, in a sense, seems to be irrelevant with respect to the angiogenic process itself. In each case, local cell death leads to release of lysosomal enzymes, which modulate basement membrane permeability or destroy basement membrane continuity, thereby mediating vascular discontinuity.

2.1.3. *Endothelial Cell Retraction*

Endothelial cell retraction is an alternate means of establishing vascular discontinuity and often occurs in combination with (or may indirectly lead to) alterations in the basement membrane architecture. Retraction can be induced by changes in pH or by endothelial cell membrane changes induced by binding with specific antibodies or lectins. It can be brought about by hyperthermia. It can be evoked by interference with the normal cell/substrate adhesion processes mediated by fibronectin, laminin, vitronectin and other cell adhesion molecules that promote cell/substrate attachment. Secondarily, endothelial cell retraction alters local conditions, such as blood flow and vessel leakiness, which, in turn, may exert a reciprocal effect on the endothelial cells or affect the matrix substrate, thereby furthering vascular discontinuity, as well as promoting the next steps in the angiogenic process.

2.2. Endothelial Cell Movement

2.2.1. *Chemokinesis*

Chemokinesis, the movement of cells in response to chemical stimuli, is an almost universal aspect of angiogenesis. Endothelial cells, normally quiescent, migrate out from previously contained blood vessels, responding both to factors in the local extravascular microenvironment or, more dramatically, to an external source of migration-inducing chemicals. There are a large number of motility-inducing factors, some of which, such as those contained in serum, show effects on many different cell types and others, such as some of the prostaglandins, that appear to act selectively on endothelial cells in particular.

2.2.2. *Chemoattractants*

Chemoattractants represent a subset of factors that induce chemokinesis, but that are operationally distinguished by the fact that they are released locally and, thus, generate gradients to which endothelial cells respond by directional migration. Directionality can be enhanced by other factors that secondarily may inactivate these chemoattractants, thereby shortening the half-life of the chemoattractant and, thus, increasing the differential between the concentration at the source of the chemoattractant and the target endothelial cells. Even small regional differences in the concentration of a chemokinesis-inducing factor can in this way lead to directional movement.

2.2.3. *Cell–Matrix Adhesion*

In addition to providing membrane stability, cell–matrix adhesion also acts as a deterrent to cell movement. A critical balance exists between adhesions essential for substrate guidance and adhesions that interfere with cell movement. Moreover, since fragments generated from specific adhesion molecules, such as laminin, collagen and fibronectin, may either inhibit or enhance cell movement, cell surface reorganization and receptor occupancy are likely to influence markedly the net effect of interactions between endothelial cells and matrix components.

2.2.4. *Divalent Cations*

Divalent cations are critically important in providing membrane stability. Externally administered chelators, such as EDTA, as well as physiological alterations in divalent ion balance, lead to membrane instability, which, in turn, triggers cell locomotion. Further, calcium-dependent

adhesion molecules (cadherins) not only regulate homotypic adhesions between endothelial cells, but also mediate interactions between leukocytes and endothelial cells, interactions that permit the release by the leukocytes of cytokines that may modulate endothelial cell movement.

2.3. ENDOTHELIAL CELL PROLIFERATION

2.3.1. *Growth Factors*

Growth factors play an important role in regulating the rate of endothelial cell proliferation. Normally, non-dividing endothelial cells achieve significant levels of proliferation during an angiogenic response, when as many as 20% of endothelial cells may enter the S-phase of the cell cycle. Autocrine, paracrine and secreted growth factors, such as the fibroblast growth factor (FGF) family, interleukins, transforming growth factors (TGFs) and epidermal growth factor, markedly influence the rate of endothelial cell proliferation.

2.3.2. *Signal Transduction*

Signal transduction is initiated once growth factors bind to their receptors on the surface of target endothelial cells. Successful induction of cell proliferation requires not only this primary interaction between growth factors and their ligands, but a complex sequence of reactions that include phosphorylation, transport, activation of transcription factors and the resultant nuclear signalling. It is only the successful consummation of all of these signal-induced tandem reactions that leads to entry of endothelial cells into the cell cycle.

2.3.3. *Cell Contact and Cell Shape*

Both cell contact and cell shape can significantly affect the endothelial cell response to growth-stimulating signals. The phenomenon of contact inhibition of growth was clearly described in the early 1950s, and the relation of cell shape to that inhibition was accurately defined two decades ago. Although as yet little is known about the precise mechanisms by which cell shape and cell contact inhibit or capacitate cell proliferative responses, the organization and reorganization of the endothelial cytoarchitecture is a fundamental element in promoting or inhibiting the cellular response to mitogenic signals.

2.4. THREE-DIMENSIONAL RESTRUCTURING

Much less is known about the factors that lead to the formation of patent vessels. Two properties of endothelial cells are essential for three-dimensional reorganization: (1) endothelial cells, even singly, have intrinsic polarity that is manifested by forming a closed loop and (2) endothelial cells have the ability to form tight junctions with other endothelial cells, thus extending this loop to generate a multicellular array leading to tube formation. Cell–matrix interactions are vital to structural integrity and vascular patterning, whereas cell proliferation is not required.

2.5. SPROUTING

As angiogenesis proceeds, further arborization may occur at the blastemal tip of the newly formed blood vessels. This secondary sprouting follows the same rules as the initial angiogenic response, beginning with the dissolution of the basement membrane and the consequent sequential events of further endothelial cell migration, additional areas of proliferation and further restructuring of the newly formed vascular sprouts. In the absence of sprouting, sprouting angiogenesis is self-limiting, and once patency of new vessels is established, the response to angiogenic stimulation is complete.

In organizing this review, we have chosen to identify inhibitors in relation to the various component parts of angiogenesis. This means that some inhibitors will be discussed more than once.

Unavoidably, it also means that we will be left with a list of inhibitors that cannot be assigned to a specific component of angiogenesis, inasmuch as there are many agents for which the target of action is not known. Within each section, we have grouped related reagents together, in order to provide some cohesion to what is rapidly becoming an unwieldy list of pharmaceuticals and biological modifiers.

3. ANTI-ANGIOGENIC FACTORS

3.1. Background and History: The Concept of Anti-Angiogenesis

Judah Folkman and colleagues (Folkman, 1971, 1972; Gimbrone *et al.*, 1972) pioneered the concept that since tumors require blood vessels in order to grow, inhibition of blood vessel development, i.e. anti-angiogenesis, would provide a strategy for preventing both the growth of primary tumors and the inhibition of metastases. The work carried out by his group and many others has demonstrated the validity of this concept (Folkman, 1985a,b; Gimbrone *et al.*, 1972; Folkman and Ingber, 1992). Moreover, given the much broader view that angiogenesis is associated with many different processes, both normal and pathological, beyond its importance for the growth and spread of neoplasms, anti-angiogenesis is now considered a valid strategy for a large array of therapeutic strategies (Mitchell and Wilks, 1992; Billington, 1991; Fan and Brem, 1992; Schweigerer and Fotsis, 1992). For example, inhibitors of vascularization may serve as anti-inflammatory agents, they may prove useful in preventing implantation, or they may find applications in reducing hyperactivity of secretory organs. Since it may well be that the relative quiescence of the cells comprising the vasculature, i.e. of vascular endothelial cells, is due to a homeostatic mechanism in which suppressor factors (inhibitors) prevent endothelial cell proliferation, understanding the nature of anti-angiogenic processes may help identify new pharmacologic agents designed to overcome natural inhibitors. These agents could be used in promoting processes such as wound healing and the establishment of tissue and organ transplants by promoting new or renewed vascular differentiation and growth.

Several thoughtful reviews and conferences have emphasized the importance of the concept of anti-angiogenesis as a strategy for development of therapies against tumors (Kerbel, 1991; Maione and Sharpe, 1990; Moore and West, 1991; Maragoudakis *et al.*, 1992; Steiner *et al.*, 1991; Denekamp, 1984a,b, 1990, 1991, 1993; Bicknell and Harris, 1992; Brem, 1992; Folkman and Ingber, 1992; Langer and Murray, 1983; Moses, 1991).

3.2. Inhibitors of Basement Membrane Degradation

3.2.1. *Protease Inhibitors*

The principal cause of basement membrane degradation is the localized release of proteases. These include serine proteases, such as urokinase plasminogen activator (uPA), and metalloproteinases, such as the collagenases and stromelysins. Consequently, inhibitors of proteases, (for example, inhibitors of plasminogen activators (e.g. PAI-1, PAI-2)) and tissue inhibitors of metalloproteinases (e.g. TIMP-1 and TIMP-2), are anti-angiogenic because they prevent the breakdown of the matrix proteins and, thus, maintain the integrity of the endothelium (Moses and Langer, 1991b; Bacharach *et al.*, 1992). It should be noted, however, that TIMP-2 may not only affect basement membrane breakdown, but act directly on endothelial cell proliferation (Murphy *et al.*, 1993). Because amiloride, a pyrazine compound, is an inhibitor of uPA, it, like TIMP-I and TIMP-2, is effective in inhibiting angiogenesis (Avery *et al.*, 1990; Alliegro *et al.*, 1993). Tumor necrosis factor (TNFα) has been shown to increase TIMP production at low concentrations, but to reduce TIMP at higher concentrations, possibly accounting for its paradoxical effects on angiogenesis (Ito *et al.*, 1990). These investigators also reported that interleukin (IL)-1 induced increased TIMP production, but presumably any anti-angiogenic effect of IL-1 is offset by angiogenesis-inducing properties, since extrinsically administered IL-I has been shown to stimulate neovascularization.

α-Thrombin is a powerful inducer of angiogenesis, and, generally, it has been assumed that this is because of its induction of fibrin. A recent study by Tsopanoglou *et al.* (1993a), however, demonstrates that phenylalanyl-propyl-arginine chloromethyl ketone-thrombin, which interferes only with the catalytic site of thrombin, abrogates the ability of thrombin to promote angiogenesis without interfering with fibrin clot formation.

Because inhibitors of urokinase are angiogenic, agents that induce such inhibitors will also be seen as effective anti-angiogenic reagents. For example, certain fragments derived from recombinant thrombospondin (TSP) have proved to be anti-angiogenic by virtue of their inducing increased levels of PAI-1 (Bagavandoss *et al.*, 1993). TSP is discussed in more detail in Sections 3.3.13 and 3.4.2. Another illustration of a reagent that indirectly leads to production of such inhibitors is TGFβ (Pepper *et al.*, 1990, 1991; Bell and Madri, 1992 as cited in Madri *et al.*, 1992; Falcone *et al.*, 1993). The action of TGFβ, however, is more complex, as seen by the fact that Pepper and his colleagues demonstrated that it also induced the synthesis of uPA mRNA. The complexity is well described by these investigators (Pepper *et al.*, 1993): **"The nature of the angiogenic response elicited by a specific cytokine is contextual, i.e. depends on the presence and concentration of other cytokines in the pericellular environment of the responding endothelial cell."**

Chick embryos were used by Takigawa *et al.* (1990b) to study metalloproteinase inhibitors. These investigators induced angiogenesis by placing polyamine-containing methyl cellulose discs (spermine, putrescine) on chick embryo yolk sac membranes, and demonstrated that the addition of either TIMP and TIMP-2 would prevent spermine-induced neovascularization.

It may be significant that this first step in angiogenesis, the disruption of the endothelial monolayer, is also a key attribute of both tumor metastasis (Liotta *et al.*, 1991; D'Amore, 1988; Moses, 1991) and inflammation. This is because both circulating tumor cells and inflammatory cells must pass through the otherwise impenetrable endothelial cell barrier, with the consequence that inhibitors of these enzymes are not only anti-angiogenic, but also act as anti-metastatic or anti-inflammatory agents. Most of the inhibitors of basement membrane degradation interfere directly or indirectly with proteolysis.

An important point, raised by Bacharach *et al.* (1992) in their analysis of protease inhibitors, is that PAI-I and uPA are both high in places such as the uterus and placenta. This would seem to contradict the anti-angiogenic role of these inhibitors, inasmuch as both the uterus and placenta show a high degree of neovascularization. **We may invoke a Yin and Yang principle here: angiogenesis inhibitors may be high in places where rapid angiogenesis is required. The loss of inhibition permits immediate and rapid angiogenesis to occur.** A similar view can be taken for the role of tumor suppressor gene function (Bouck, 1990, 1993; Brem and Klagsbrun, 1993). Folkman (1992), referring to tumor angiogenesis in particular, states the concept clearly: **"Accumulating evidence indicates that for most tumors, the switch to the angiogenic phenotype depends on the outcome of a balance between angiogenic stimulators and angiogenic inhihitors, both of which may be produced by tumor cells and perhaps by certain host cells."**

3.2.2. *Cartilage-Derived Inhibitors*

The first reported biological inhibitor of neovascularization was cartilage, shown by Eisenstein *et al.* (1973) to inhibit blood vessel development in the chick chorioallantoic membrane (CAM) and by Brem and Folkman (1975) to abrogate V-2 carcinoma-induced angiogenesis in the rabbit cornea. The effect of cartilage was attributed, in part, to its anti-collagenolytic activity (Langer *et al.*, 1976; Kuettner *et al.*, 1977; Langer *et al.*, 1980; Kuettner and Pauli, 1983). Sorgente and Dorey (1980), as well as Takigawa *et al.* (1985, 1987), showed that cartilage also contained other molecules that inhibit endothelial cell proliferation. Additionally, a single antiangiogenic protein, cartilage-derived inhibitor (CDI), identified by Moses *et al.* (1990) as an anti-collagenase based on sequence similarity, is capable of inhibiting both endothelial cell migration and endothelial cell proliferation of capillary endothelial cells (Takigawa *et al.*, 1990c). Subsequently Moses *et al.* (1992) described a collagenase inhibitor released by chondrocytes *in vitro* (chondrocyte-derived inhibitor) which was anti-angiogenic in the chick embryo (CAM) assay, but which also showed anti-migratory and anti-proliferative effects when added to basic fibroblast growth factor (bFGF)-stimulated capillary endothelial cells.

There has been much recent discussion of the possibility of utilizing shark cartilage as a source of anti-angiogenic activity. Although there is a considerable question concerning the efficacy of commercial derivatives of shark cartilage, the initial finding that shark cartilage manifests anti-angiogenic activity (Langer *et al.*, 1976) seemed to be correlated with its protease-inhibitory activity (Lee and Langer, 1983). Recently, a low molecular weight fraction obtained from crude shark cartilage (MW 10^3–10^4), resistant to heat treatment, was identified by Oikawa and colleagues (1990a). The size and heat stability of this derivative places it in a different molecular category from the known protease inhibitors previously identified in shark cartilage.

It immediately becomes apparent that the assignment of a reagent to one or another phase of the angiogenic process is complicated by the fact that an action on an early phase of the process, such as membrane degradation, may be manifested in an assay that measures a subsequent step, such as endothelial cell movement, proliferation or tube formation. In addition, some reagents may have more than one action, in some cases based on different domains in their molecular structure, sometimes by having varied effects on different cellular targets, or quite frequently, for reasons that are as yet undetermined.

3.2.3. *Epithelium-Derived Inhibitors*

The identification of inhibitors in cartilage resulted from the recognition that cartilage is normally avascular (Eisenstein *et al.*, 1973). A similar argument was made to explain the resistance of epithelia to vascularization (Waxler *et al.*, 1982), and the validity of this suggestion was demonstrated by identifying protease inhibitory activity in urinary bladder epithelium. It is surprising that relatively little research has been directed at exploiting avascular eptihelia as a source of potentially useful anti-angiogenic factors.

3.2.4. *Phorbol Esters*

This problem of the complex action of anti-angiogenic agents is seen very clearly in studies of tumor promoting agents such as phorbol myristate acetate (PMA). Most tumor promoters are potent stimulators of collagenase and plasminogen activator, and their ability to induce angiogenesis has been ascribed to this property (Montesano and Orci, 1985; Montesano, 1992). Not surprisingly, therefore, they found that PMA-induced angiogenesis, measured *in vitro* by induction of cell movement into collagen gels to form tubes, could be inhibited by 1–10-phenanthroline, a metalloprotease inhibitor. As discussed in Section 3.4.3, however, Doctrow and Folkman (1987) found that tumor promoters themselves may augment or inhibit endothelial cell proliferation, with differing effects depending on the type of endothelial cell target. Other collagenase inhibitors, such as certain sulfated chitin derivatives, are anti-angiogenic *in vivo*, preventing initiation of new vessels, and anti-migratory (but not anti-proliferative) *in vitro* (Murata *et al.*, 1991), suggesting that these compounds may also act on more than one step in the angiogenic process.

3.2.5. *Steroids*

Gross *et al.* (1981) used the rabbit cornea assay to demonstrate anti-angiogenic properties of medroxyprogesterone acetate, an angiostatic steroid that Ashino-Fuse *et al.* (1989) identified as an inhibitor of plasminogen activator. We should note, however, that Tokida *et al.* (1990) have reported that medroxyprogesterone also changes the subunit structure of laminin, and attribute at least part of the effect of angiostatic steroids to their ability to alter, rather than destroy, the basement membrane. Wolff *et al.* (1993) reported on anti-angiogenic effects of dexamethasone, correlating their results with a concomitant reduction of plasminogen activator activity. Blei *et al.* (1993) also examined the effect of medroxyprogesterone, testing the effect of this steroid on cultured bovine aortic endothelial cells grown in the presence of bFGF. They also ascribe the angiostatic effect to the reduction of plasminogen activator, which they attribute to steroid-induced increase in PAI-1. However, dexamethasone may exert its effect through an alternate or additional pathway. Takatsuka *et al.* (1992) reported that dexamethasone produced its anti-angiogenic effect by inhibiting FGF-induced angiogenesis as measured in the cornea. This alternate route of action may

also be true for medroxyprogesterone. Fujimoto *et al.* (1989a) extended the results with medroxyprogesterone to the inhibition of angiogenesis by subcutaneously growing mammary tumors. These investigators (Fujimoto *et al.*, 1989b; Jikihara *et al.*, 1992), found that the ability of medroxyprogesterone to inhibit angiogenesis in gynecologic tumors could be attributed to its inhibition of bFGF.

Oikawa *et al.* (1988) proposed that the ability of medroxyprogesterone acetate to inhibit carcinogen-induced tumor growth was due to its anti-angiogenic action, but did not address the mechanism of inhibition. Angiogenesis in the posterior segment of the eye could also be inhibited by steroids, as shown by Antoszyk *et al.* (1993), who introduced triamcinolone acetonide into the vitreous to inhibit inflammatory retinal neovascularization.

Ingber *et al.* (1986) used the explanted chick embryo CAM to assess the effect of angiostatic steroids and heparin, noting that when these agents were administered locally in methylcellulose discs, they led to capillary basement membrane breakdown, capillary retraction and regression. These investigators then expanded their analysis to other modulators of matrix components (Ingber and Folkman 1988, 1989), reporting that proline analogs and *trans*-retinoic acid, both of which inhibit collagen accumulation, augmented the steroid/heparin effect and could lead to complete inhibition of new, as well as regression of already-formed, CAM capillaries.

Gagliardi and Collins (1993) also used the chick CAM assay system, and were able to demonstrate that avascular areas were generated around implanted methylcellulose discs containing a variety of antiestrogens, including clomiphene, tamoxifen, nafoxidine, and two steroidal anti-estrogens (ICI 182; ICI 780). However, they advise about drawing conclusions concerning these anti-estrogens, since addition of excess estrogen (17β-estradiol) did not alter the anti-angiogenic effect of these inhibitors.

How analogues of somatostatin inhibit angiogenesis is not known (Woltering *et al.*, 1991), although it can be suggested that the angiostatic properties of these compounds are related to those of the steroids discussed above (see also Grant, M. B. *et al.*, 1993a; Barrie *et al.*, 1993).

3.2.6. *Antibiotics*

Tamargo *et al.* (1991) ascribed the anti-angiogenic effect of minocycline, measured by its inhibition of angiogenesis by V2 carcinoma tissue implanted into the rabbit cornea, to the anti-collagenase activity of this antibiotic (see also Teicher *et al.*, 1992; Guerin *et al.*, 1992).

Sulphonated derivatives of distamycin A were synthesized to determine whether these would be able to complex with various growth factors, such as bFGF and platelet-derived growth factor-β (Mariani *et al.*, 1992). Several of these compounds were found to be anti-angiogenic on the CAM, prompting the studies of Sola *et al.* (1992), which led to the demonstration that these inhibitors were able to exert a significant inhibitory effect on the growth of the bFGF-producing M5076 tumor *in vivo*.

3.3. INHIBITORS OF CELL MIGRATION

There are a number of ways in which an agent can affect endothelial cell migration: (1) it may act on endothelial cells directly; (2) it may interfere with migration-inducing or migration-augmenting factors; (3) it may alter the tissue microenvironment so that endothelial cell movement is no longer possible.

3.3.1. *Taxol, Colchicine, Vinblastine, Nocodazole*

The most direct method is to target the endothelial cell itself: to interfere with the ability of an endothelial cell to effect the internal structural reorganization essential for achieving motility. For example, compounds such as taxol (Coomber and Gotlieb, 1990), colchicine, vinblastine or nocodazole, which limit cytoskeletal reorganization, decrease or completely prevent endothelial cell movement (Schwartz *et al.*, 1979 as cited in Coomber and Gotlieb, 1990). The fact that taxol is inhibitory at low concentrations is significant because even at concentrations too low to interfere with proliferation, it may interfere with motility. Since cell movement requires significant

expenditure of energy, any agent that reduces the metabolic activity of endothelial cells will affect the ability of these cells to migrate.

3.3.2. *Interferons*

Another agent that was shown to act directly on endothelial cell movement is IFN. Since the early report by Brouty-Boye and Zetter (1980) that leukocyte (α/β) IFN inhibits capillary endothelial cell motility *in vitro*, the anti-angiogenic effects of recombinant IFNs have been reported (Sidky and Borden, 1987; Sato, N. *et al.*, 1990) and IFN-α has become an accepted therapy for inducing regression of hemangioendotheliomas (Orchard *et al.*, 1989; Ezekowitz *et al.*, 1992) and possibly of other hemangiomatous disease states (White *et al.*, 1989). Guyer *et al.* (1992) have proposed that because choroidal neovascularization is at present untreatable, controlled randomized trials of IFN-α therapy for this disease manifestation should be undertaken. This suggestion is all the more valid since Miller *et al.* (1993) recently showed that experimental iris neovascularization induced in monkeys was inhibited and already-induced blood vessels regressed following systemic administration of IFN-α. Engler *et al.* (1993), however, in their study of five patients with subfoveal neovascular macular degeneration, caution that IFN-α results are not uniformly successful and emphasize the importance of carrying out controlled studies on a larger number of patients before accepting IFN-α as a useful anti-angiogenic agent for this disease. Poliner *et al.* (1993) suggest similar reservations, since no long-term benefit of IFN-α2a therapy was seen in their study of 19 additional patients with subfoveal neovascularization associated with age-related macular degeneration. Whether IFN-α therapy might be useful for other angiogenic diseases remains to be determined.

One possible explanation for diverse results with IFN-α may lie in the fact that IFN-α may potentiate the angiogenic effect of IL-2 (Cozzolino *et al.*, 1993), both as measured in the rabbit cornea and in *in vitro* proliferation assays. Their studies indicate that as a result of the combined action of IFN-α and IL-2 there is an increase both in the synthesis and in the release of bFGF, this in turn increasing the rate of proliferation of cultured human umbilical vein endothelial (HUVE) cells. The role of IFNs in regulating endothelial cell proliferation and tube formation is discussed in Sections 3.4.6 and 3.5.2.

3.3.3. *Cholera Toxin*

Based on extensive studies of the importance of gangliosides in promoting cell movement and metastasis, Alessandri *et al.* (1986, 1992) examined the effect of cholera toxin, known to bind to GM_1. When tested in a transmembrane migration chamber assay (Boyden chamber), cholera toxin proved to be inhibitory to migration of bovine adrenal capillary endothelial cells. However, the effective dose needed to inhibit migration induced by GM_1 was high, so that even 100 μg/mL was only partially effective. Interestingly, these investigators also noted the anti-migration effects of IFN-γ (see Section 3.3.2), and ascribed these to the ability of IFN-γ to block gangliosides. The intricate nature of ganglioside regulation of angiogenesis was illustrated by the observation that changes in the ganglioside ratio of GM3/GD3 could lead to augmentation or retardation of angiogenesis induced in the cornea by prostaglandin E_1 or bFGF (Ziche *et al.*, 1992).

3.3.4. *The TGFβ Family*

The role of members of the TGFβ family is complex. The TGFβs are widely distributed, as well as developmentally regulated, and it has been suggested that they act in multiple ways in both a paracrine and autocrine manner (Pelton *et al.*, 1991). As mentioned earlier (Section 3.3.1), TGFβ can induce inhibitors of PAI activators and thereby affect the initiation of angiogenesis. TGFβ also inhibits endothelial cell migration, as shown for bovine heart, adrenal and corneal endothelial cells by Müller *et al.* (1987). More recently, Madri *et al.* (1992) showed that both TGFβ1 and TGFβ3 isoforms affect endothelial cell migration, inhibiting the movement of bovine aortic endothelial cells. On the other hand, Yang and Moses (1990) analyzed TGFβ1-induced angiogenesis on the CAM, and concluded that both capillary cell migration and proliferation were *increased* by

exposure to TGFβ. TGFβ may also have an indirect effect on angiogenesis, because its ability to inhibit E-selectin expression on endothelial cells (Gamble *et al.*, 1993a,b) may reduce paracrine effects of lymphocytes by preventing their adhesion to the endothelium (see also Bereta *et al.*, 1992). Because TGFβ is primarily considered to be an inhibitor of endothelial cell growth, much of the discussion of the action of TGFβ is deferred to Section 3.4.5. **But the studies on cell migration already accentuate an important question—the difficulty of correlating selective *in vitro* reactions of endothelial cells with the complex total manifestation of new blood vessel formation *in vivo*.**

3.3.5. *α-Difluoromethyl Ornithine and Other Inhibitors of Ornithine Decarboxylase*

Pleiotropic action on both movement and growth of endothelial cells is also seen for inhibitors of ornithine decarboxylase. We have already discussed the effect of polyamines in angiogenesis, and their inhibition by tissue inhibitors of metalloproteinases. However, α-difluoromethylornithine, which irreversibly inhibits ornithine decarboxylase, also inhibits polyamine-induced angiogenesis, as evidenced in the chick CAM assay, and this effect correlates with the inhibition of migration, as well as proliferation of bovine pulmonary artery endothelial cells *in vitro* (Takigawa *et al.*, 1990a). α-Difluoromethylornithine is an effective inhibitor of both tumor-induced and lymphocyte-induced angiogenesis *in vivo* (Monte *et al.*, 1993).

3.3.6. *Inhibitors of FGF: Protamine, PF4, Suramin*

A second means of inhibiting endothelial cell movement is specific to the agent that induces or augments cell movement. The best studied motility-inducing agent has been bFGF, while heparin was one of the earliest and most analyzed molecules demonstrated to augment FGF-induced endothelial cell movement. Not surprisingly, therefore, antagonists of FGF, such as antibodies to FGF (Hori *et al.*, 1991) prevent endothelial cell movement. Similarly, protamine, known for its ability to bind to heparin, markedly inhibited new blood vessel formation in the chick embryo (Taylor and Folkman, 1982; Tanaka *et al.*, 1986), a finding made all the more significant by the fact that protamine had no effect on non-growing, mature vessels (see note 12 in Taylor and Folkman, 1982; Jakobsson *et al.*, 1990; but cf. Neufeld and Gospodarowicz, 1987). The basis for the protamine-induced inhibition of myocardial angiogenesis, reported by Flanagan *et al.* (1991) has not been determined, but may be due to inhibition of both migration and proliferation (see also Section 3.4.1). Platelet Factor 4 (PF4) has been assumed to act in a similar fashion because of its molecular similarity to protamine, and this was demonstrated experimentally by its effect on bovine aortic endothelial cell movement in an *in vitro* "wounding" assay (Sato, Y. *et al.*, 1990, 1993b) as well as in a random migration assay (Sharpe *et al.*, 1990). In these studies there was a direct correspondence between the extent of inhibition and the binding of bFGF to its receptor. The anti-angiogenic effect of suramin can also be attributed partly to its antagonistic action to heparin (Gagliardi *et al.*, 1992). However, the action of suramin is doubtless more complex, since it has been demonstrated that suramin is a potent inhibitor of several DNA- and RNA-polymerases (Ono *et al.*, 1988). In addition, suramin competes for the vasculotropin receptor on bovine adrenal capillary endothelial cells (Plouet and Moukadari, 1990). Furthermore, suramin has been shown to inhibit cell responses to a variety of cytokines with angiogenic activity including IL1β and IL-6 (Baumann and Strassman, 1993).

3.3.7. *Corticosteroids and Heparin*

A differential effect of hydrocortisone in combination with a heparin analog, hexosaminoglycan sulfate, on the production of migration-inducing factors by several types of tumors was reported by Rong *et al.* (1986). Whereas neither hydrocortisone alone nor the heparin analog alone had any detectable influence on the production of angiogenesis or migration-inducing factors by any of several tumor cell lines, the combination reduced factor production by three of four tumor lines, as monitored both by angiogenesis in the corneal assay and by *in vitro* migration of bovine adrenal capillary endothelial cells in a Boyden chamber. J. K. Lee *et al.* (1990) examined the growth of cells from a human neurofibrosarcoma transplanted into athymic mice, finding that the combi-

nation of heparin and hydrocortisone was effective *in vivo* as well, whereas heparin alone actually stimulated angiogenesis. On the other hand, another study showed that cortisone acetate, even without heparin, was effective *in vivo* in reducing tumor-induced angiogenesis induced in the dorsal air sac hy a murine bladder tumor cell line (Lee, K. *et al.*, 1987; but cf. Sakamoto *et al.*, 1986). Moreover, K. E. Lee *et al.* (1993) observed that cortisone acetate was effective in inhibiting the growth rate of these bladder tumor cells when these were inoculated intradermally into syngeneic mice.

Thorpe *et al.* (1993) recently generated a heparin–steroid conjugate in which a non-coagulant derivative of heparin was linked to hydrocortisone. The ability of this conjugate to affect endothelial cell motility was measured both in a monolayer wounding assay system utilizing mouse microvascular endothelial cells and in a sponge implant system. Inhibition of migration was virtually complete in the monolayer assay, and blood vessel formation in the sponges was markedly delayed or completely abolished.

The interactions between steroids with heparin and heparin-like compounds are discussed further in the Section 3.4.7.

3.3.8. *Interleukin-8*

One of the most potent angiogenesis-inducing cytokines is IL-8, one of the angiogenic molecules released by macrophages. Human recombinant IL-8 induces both chemotaxis and proliferation of HUVE cells, as well as eliciting corneal angiogenesis. Antibodies to IL-8 were able to block the angiogenic (chemotactic) activity of both synovial fluid tissue macrophages and of stimulated monocytes (Koch *et al.*, 1992; see also Elner *et al.*, 1991). It is particularly interesting that an IL-8 antisense oligonucleotide could specifically prevent the production of angiogenic factors by monocytes *in vitro* (Koch *et al.*, 1992). Cohen *et al.* (1982), however, had shown previously that other cytokines, not identified at the time, may act directly to inhibit endothelial cell migration.

3.3.9. *SPARC*

Sage and colleagues have carried out an extensive analysis of SPARC ("Secreted Protein, Acidic and Rich in Cysteines"), an extracellular, Ca^{2+}-binding glycoprotein, associated with cell migration, proliferation and differentiation of various cell types (Funk and Sage, 1991; Sage, 1992). Recently, Hasselaar and Sage (1992) demonstrated that in the absence of serum, SPARC was able to inhibit bFGF-induced migration of endothelial cells both in Boyden chambers and in the *in vitro* monolayer repair (wound healing) model of angiogenesis. Interestingly, SPARC did not inhibit motility of endothelial cells in the presence of serum, suggesting that SPARC interacts with one or more serum factors in the regulation of cell migration.

3.3.10. *Inhibitors of Platelet-Activating Factor*

Smither and Fan (1992), as well as Vieria *et al.* (1992) describe studies that document the complex pro-antiogenic activity of platelet-activating factor (PAF), including its ability to induce collagenase production, increase endothelial cell migration, increase vascular permeability and augment endothelial cell proliferation. In their report, they cite experiments documenting that three structurally unrelated PAF antagonists inhibit angiogenesis in their *in vivo* sponge model assay. Similarly, Vieria *et al.* (1992) describe anti-angiogenic effects of *Bothrops jararaca* venom, and attribute these effects to the venom's ability to counteract the positive angiogenic effect of PAF.

3.3.11. *Targeting Mast Cells and Macrophages: Thiols and Gold-Containing Compounds*

A third means of reducing or inhibiting endothelial cell movement is indirect, targeting, not the endothelial cell itself, but an intermediary effector cell. This indirect effect, already mentioned in our discussion of IL-8, is most readily seen in inflammatory angiogenesis, where the role of mast cells and macrophages is particularly well documented. Mast cells, when activated, release a variety of vascular mediators, including histamine and heparin, both of which *promote* endothelial cell

locomotion (cf. Folkman, 1982). However, they also release TNFα (Gordon and Gall, 1990; Manning *et al.*, 1992), which can *inhibit* endothelial cell motility as well as proliferation (Sato *et al.*, 1986; Mano-Hirano *et al.*, 1987; Schweigerer *et al.*, 1987). It is noteworthy that TNF was able to completely inhibit migration of bovine adrenal capillary endothelial cells in response to tumor extracts, but that it did not inhibit spontaneous migration of these cells (Mano-Hirano *et al.*, 1987). TNF is discussed more fully as an inhibitor of endothelial cell proliferation in Section 3.4.6.

The anti-angiogenic effect of thiol-containing compounds and various gold-containing anti-rheumatic compounds have been ascribed, at least in part, to their effect on macrophages, rather than their direct effects on endothelial cells (Koch *et al.*, 1988, 1991). This explanation cannot be the only mechanism by which gold compounds achieve their effects, however, since Matsubara and Ziff (1987) were able to demonstrate a direct antiproliferative effect on endothelial cells *in vitro*.

3.3.12. *Targeting Lymphocytes: Steroids, Anti-Lymphocyte Sera, Irradiation*

Since stimulated lymphocytes are potent inducers of angiogenesis (Sidky and Auerbach, 1975), primarily due to their release of migration-inducing lymphokines (Auerbach, 1981; Obeso *et al.*, 1990; Auerbach *et al.*, 1991b), any treatment that reduces lymphocyte numbers or lymphokine production can reduce the migratory component of angiogenesis. The effect of reducing immuno-competent lymphocytes on angiogenesis is most readily seen in the studies of Suvarnamani *et al.* (1989), who reported that total lymphoid irradiation materially suppresses corneal neovascularization induced by silver/potassium nitrate cauterization.

The anti-angiogenic effect of cyclosporin (Norrby, 1992; Lipman *et al.*, 1992) may also be attributed to its abrogation of lymphocyte function. A similar indirect inhibitory effect has also been proposed for opioids such as β-endorphin or morphine sulfate, both of which reduce blood vessel formation on the chick CAM (Pasi *et al.*, 1991). A marked reduction in angiogenesis has also been observed when allogenic lymphocytes are injected into tumor-bearing mice (Sidky and Auerbach, 1976), but whether this inhibition is due to an effect of the tumor on the effector lymphocytes or on the host response to those lymphocytes has not been established.

AGM-1470, a synthetic variant of a fungal antibiotic, has been shown to inhibit T-cell proliferation in response to mitogenic signals such as tetanus toxoid (Berger *et al.*, 1993). To what extent the powerful anti-angiogenic action of AGM-1470 involves T-cell inhibition, however, has not been determined. AGM-1470 is discussed more fully in Section 3.4.13.

3.3.13. *Targeting the Extracellular Matrix: Peptides, Antibodies, Sulfated Chitin Derivatives*

A fourth set of reagents acts to reduce or inhibit endothelial cell movement by indirect means aimed at altering the matrix or tissue environment in which cell migration must take place. Endothelial cells migrate over a matrix of fibronectin, collagen, vitronectin, laminin and glycoconjugates, such as TSP and syndecan. Alterations in cell/matrix adhesion, e.g. blocking of adhesion sites by soluble peptides (Nicosia and Bonanno, 1991; Saiki *et al.*, 1990a; Sakamoto *et al.*, 1991), or modulating endothelium-associated cell surface ligands by antibodies will lead to an inhibition of endothelial cell movement. Similarly, antibodies against TSP will prevent the migration of endothelial cells into an *in vitro* denuded "wound" area of an otherwise confluent culture plate (Alessio *et al.*, 1991). Taraboletti *et al.* (1990) obtained paradoxical results with soluble TSP. At low concentration, it was found to enhance bFGF-induced cell migration, while at high concentration, it was inhibitory. To what extent the demonstration that certain fragments of TSP are more inhibitory to motility and metastatic spread of tumor cells derives from their effect on endothelial cells (Yabkowitz *et al.*, 1993; Bagavandoss *et al.*, 1993) remains to be determined.

Because matrix molecules, such as fibronectin and TSP, have binding sites for heparin, they may also secondarily affect other processes, especially proliferation of endothelial cells (see Section 3.4), which rely on the ability of growth factors to bind to heparin (cf. Homandberg *et al.*, 1985, 1986). Other agents that may affect cell migration through their effects on matrix molecules are the sulfated chitin derivatives (Saiki *et al.*, 1990b; Murata *et al.*, 1991), as well as the already mentioned cartilage- and chondrosarcoma-derived inhibitors (Moses and Langer, 1991a; Moses *et al.*, 1992). Agents that inhibit synthesis of matrix molecules either directly or indirectly thus can be classed

as anti-angiogenic. However, we will not, in this review, discuss general inhibitors of protein synthesis, except as they reflect findings of special importance to the angiogenic process.

3.3.14. *Heparin*

The question of heparin binding is of special interest, because heparin (and heparin fragments) have been shown both to augment and to inhibit angiogenesis *in vivo* and endothelial cell migration and proliferation *in vitro* (Folkman, 1985c; Rosenbaum *et al.*, 1986; Obeso *et al.*, 1990; Klein-Soyer *et al.*, 1992; Norrby, 1993a; see Freedman, 1992 for overview). The largest family of angiogenic agents is the heparin-binding growth factor family. Bound heparin facilitates growth factor action. On the other hand, excess soluble heparin or heparan sulfate can act as competitive inhibitors of binding of the heparin-binding growth factors to the extracellular matrix, reducing the efficacy of these growth factors (Hahnenberger and Jakobson, 1991; Jakobson and Hahnenberger, 1991). An interesting additional point was raised by Ihrcke *et al.* (1993), who suggest that heparan sulfate released from endothelial cells may either up-regulate or down-regulate cellular immune responses by modulating the interaction of antigen-presenting cells and T-cells. Thus, soluble heparan sulfate indirectly may lead to changes in the production of angiogenic or angiostatic cytokines.

Using an *in vivo* experimental system in which cells are permitted to migrate into and proliferate within an implanted polyvinyl sponge (Fajardo *et al.*, 1988a), Kowalski and coworkers (1992) demonstrated that heparin alone could induce cell migration but not cell proliferation, in contrast to its action in combination with bFGF, where heparin promoted cell proliferation as well. It will be important to determine whether the distinction between effects of heparin on cell migration and cell proliferation may reflect the differential roles of low molecular weight and high molecular weight fractions of heparin (Norrby, 1993a).

3.3.15. *Prostaglandins*

Prostaglandins E_1 and E_2 are able to increase endothelial cell motility (cf. Ziche *et al.*, 1985), and it is this property that appears to be responsible for their angiogenic effects both on the chick CAM and in the rabbit cornea. Just as inhibition of cell movement may be one of the means by which agents, such as antagonists of FGF, produce their anti-angiogenic effects (Robertson *et al.*, 1991; Rong *et al.*, 1986), inhibitors of prostaglandin synthesis, like indomethacin and aspirin (Peterson, 1983, 1986), ketorolac (Kowalski *et al.*, 1992), mitoxantrone or bisantrene (Polverini and Novak, 1986), α-guiaconic acid and their derivatives (Ito *et al.*, 1993), and other proprietary compounds (Haynes *et al.*, 1989, 1992) may have anti-angiogenic consequences. We have already assigned the anti-angiogenic effect of amiloride to its inhibition of uPA. However, Avery *et al.* (1990) also showed that amiloride was able to inhibit prostaglandin-induced angiogenesis. The selective nature of this type of inhibition lies in the fact that while such inhibitors are effective against FGF-induced angiogenesis, they leave unaffected endothelial cell responses to growth factors other than FGF (see also Fotsis *et al.*, 1994). Selectivity has both advantages and disadvantages when designing anti-angiogenic approaches. For example, if a particular tumor induces blood vessels by release of FGF or FGF-related factors, these agents will inhibit blood vessel formation in that tumor, yet leave intact alternate means of achieving vascular proliferation, wound healing, etc. On the other hand, a different tumor, which induces neovascularization by an alternate route (e.g. VEGF), will not respond to the anti-angiogenic effect of agents that work through their inhibition of FGF action.

3.3.16. *Placental Ribonuclease Inhibitor*

Using the disc angiogenesis assay developed by Fajardo *et al.* (1988a), Polakowski *et al.* (1993) demonstrated that RNasin, a recombinant form of placental ribonuclease inhibitor, inhibits migration of endothelial cells into the polyvinyl sponge implant. Although the assumption is that the effect of the inhibitor is directed against angiogenin (cf. Shapiro and Vallee, 1987, 1991; Badet *et al.*, 1990), a non-heparin-binding protein that activates endothelial cells, the role of this inhibitor may be more complex because RNasin is also inhibitory to several angiogenic agents, such as bFGF

and sodium orthovanadate, which exhibit no obvious relationship to angiogenin. Moreover, placental ribonuclease inhibitor fails to compete with the angiogenin-binding protein expressed on endothelial cells (Hu *et al.*, 1991). On the other hand, the binding of placental ribonuclease inhibitor to angiogenin is tight, with 1 : 1 stoichiometry, in contrast to a much weaker affinity of the inhibitor to other ribonucleases (Lee *et al.*, 1989). If, then, the inhibitor affects angiogenesis induced by agents other than angiogenin, it may be that angiogenin is a participant in some stage(s) of the angiogenic process subsequent to the action of FGF or sodium orthovanadate. The finding by Soncin (1992) that endothelial cell adhesion to angiogenin-coated plastic is inhibited by the soluble glycine–arginine–glycine–asparagine–serine (GRGDS)- containing peptide known to block integrin receptor interactions supports this interpretation. The latter interpretation is also supported by the finding of an anti-angiogenic effect of a mutated form of angiogenin (Shapiro and Vallee, 1989). Using site-specific mutagenesis to replace either of two critical histidines by alanine, these investigators generated variant molecules that inhibited the angiogenic activity of angiogenin, as tested in the CAM assay, although they did not inhibit binding of angiogenin to the endothelium. As more information is gained from site-directed mutagenesis analysis of angiogenin (Shapiro and Vallee, 1992), it should be possible to generate specific inhibitors that target those moieties on angiogenin essential for its angiogenic activity.

It now appears that the ribonuclease/angiogenin inhibitor is part of a larger superfamily of molecules with repetitive leucine-rich motifs, among which are molecules conserved across wide species divergence. For example, the yeast RNAI protein essential for RNA processing shows strong similarity to the placental ribonuclease inhibitor (Schneider and Schweiger, 1992). It remains to be determined whether this yeast homologue, as well as other members of the superfamily, may manifest anti-angiogenic activity in mammalian systems.

A recent report by Hu *et al.* (1993) identifies actin as an angiogenin-binding protein. It is most interesting, therefore, that both actin and an anti-actin antibody inhibit angiogenin-induced angiogenesis in the CAM assay. **This finding illustrates an important principle: anti-angiogenic action can be directed both at angiogenic molecules or their receptors, i.e. interference with effector molecule/ligand binding can block the triggering of an angiogenic response.**

3.3.17. *Antibiotics*

Oikawa and colleagues reported on anti-angiogenic properties of several antibiotics, including herbamycin (Oikawa *et al.*, 1989b), bleomycin (Oikawa *et al.*, 1990b), eponemycin (Oikawa *et al.*, 1991), erbstatin (Oikawa *et al.*, 1993a) radicicol (Oikawa *et al.*, 1993b) and staurosporine (Oikawa *et al.*, 1992b), as tested in the chick CAM assay system. In the eponemycin studies, they included assessment of the effect of the antibiotic on cultured bovine carotid artery-derived endothelial cells, reporting its interference with both cell migration (Boyden chamber assay) and proliferation (Oikawa *et al.*, 1991). The anti-angiogenic activity of both eponemycin and staurosporine was attributed to their inhibition of protein kinases.

3.3.18. *Other Inhibitors of Cell Migration*

Nicardipine, a dihydropyridine used clinically as a calcium-channel blocker, was tested for its ability to inhibit *in vitro* migration, proliferation and tube formation of bovine aortic endothelial cells (Kaneko *et al.*, 1992). Its effect on migration was impressive, inhibition was shown to be in a dose-dependent manner, with a dose as low as 10^{-8} M inhibiting transwell migration by 50%. Nicardipine had no effect on proliferation, whereas its ability to inhibit tube formation (discussed in Section 3.5.7) was incomplete and required considerably higher concentrations of the drug. Among the retinoids, the synthetic compound fenretinide was found to inhibit aortic endothelial cell motility, as measured in the phagokinetic track assay (Pienta *et al.*, 1993).

Retinoids are discussed more fully in their role as inhibitors of endothelial cell proliferation in Section 3.4.4.

There a number of inhibitors of cell migration that have not been specifically tested for their anti-angiogenic action. For example, sphingosine-1-phosphate, one of several sphingolipids analyzed for anti-tumor action, was found to inhibit tumor cell motility in a phagokinetic track

assay, as well as migratory activity across a Matrigel-coated membrane (Sadahira *et al.*, 1992). One of the observations made was that this compound inhibited 3T3 cell migration, but not the migration of bovine pulmonary artery endothelial cells. This finding is intriguing in that 3T3 cells are frequently used as a model for angiogenic and anti-angiogenic effects. Although the authors do not discuss this finding further, it seems important to consider the possibility that sphingosine-1-phosphate may act selectively on microvascular endothelial cells. The fact that this compound appears to affect the actin filament organization identified as critical to the action of angiogenin (cf. Soncin, 1992) further strengthens this suggestion.

Linomide (*N*-phenylmethyl-1,2-dihydro-4-hydroxyl-1-methyl-2-oxoquinoline-3-carboxamide), a quinolinecarboxamide long known as an immunomodulator and anti-tumor agent, was also described recently as being anti-angiogenic (Vukanovic *et al.*, 1993). Among its effects *in vitro* were a general cytostatic effect on endothelial cells and a specific inhibitory effect of endothelial cell migration measured in Boyden chambers. It also inhibits angiogenesis *in vivo* as measured in the Matrigel assay of Passaniti *et al.* (1992).

As yet, there is only fragmentary information on the extent to which platelet-endothelial cell adhesion molecule-1 (PECAM-1), which is constitutively expressed on endothelial cells, affects angiogenesis. Recently, Schimmenti *et al.* (1992) have transfected PECAM-1 into NIH/3T3 cells and found that these transfectants showed reduced cell migration in a monolayer migration assay system. These studies serve as a prototype for the assessment of anti-migratory effects of a plethora of cell adhesion molecules, some of which, like PECAM-1, are expressed constitutively, whereas others are induced by a variety of cell activating factors such as IL-1, IFN-γ or bacterial lipopolysaccharide.

3.4. Inhibitors of Endothelial Cell Proliferation

By far, the largest number of studies on anti-angiogenesis have assessed the ability of various agents to inhibit endothelial cell proliferation. The reasons for this are both conceptual and practical. From a conceptual standpoint, it is recognized that even if angiogenesis can be initiated and vessel sprouting and endothelial cell movement are induced, the amount of new vessel formation will be small unless there can be a replacement and augmentation of those endothelial cells that have pushed beyond their initial confines. From a practical standpoint, cell proliferation can be assessed rapidly, is readily quantifiable, and permits the use of established cell lines.

3.4.1. *Inhibitors of Fibroblast Growth Factor*

One of the first factors identified as promoting endothelial cell growth was FGF. It became the prototype of a large number of heparin-binding growth factors. Although we have already discussed FGF as a promoter of cell migration, it is as a growth factor that it has received the most attention. (Parenthetically, the fact that FGF promotes the growth of *fibroblasts* deserves emphasis: **Almost without exception the growth-promoting factors studied experimentally exert their effect not only on endothelial cells but on many other cells as well. Thus, the description of anti-angiogenic agents that modulate growth must also be evaluated from the standpoint of specificity of target cell action.)**

Not unexpectedly, blocking antibodies to FGF will inhibit endothelial cell proliferation (Sakaguchi *et al.*, 1988; Matsuzaki *et al.*, 1989; Reilly *et al.*, 1989) just as they do FGF-induced migration (see Section 3.3.6). Matsuski and colleagues report, however, that the same antibodies do not necessarily inhibit angiogenesis, since anti-FGF-producing hybridomas transplanted into athymic mice became highly vascularized even in the presence of high levels of blocking antibodies to FGF. There are two, not necessarily mutually exclusive, explanations for these observations: (1) the myelomas may induce angiogenesis by release of factors other than FGF; (2) FGF may be released locally in a form that cannot be accessed by the antibody or it may be bound to receptors before the antibody can block its action.

Compounds that compete with heparin or heparan sulfate for the binding of growth factor, such as FGF, are, as expected, anti-angiogenic. This can be well illustrated by the observation that pentosan polysulfate inhibits heparin-binding of Kaposi's sarcoma-derived FGF (Zugmaier *et al.*, 1992; Wellstein *et al.*, 1991). The ability of heparinase to inhibit neovascularization similarly has

been attributed to its interference with the interaction between heparin and bFGF (Sasisekharan *et al.*, 1994).

As mentioned in Section 3.3.6, the anti-angiogenic effect of protamine was based, in part, on its inhibition of FGF-induced cell movement. However, when Neufeld and Gospodarowicz (1987) added protamine sulfate to freshly seeded cultures of bovine aortic endothelial cells, they found that acidic FGF (aFGF) and bFGF-induced cell proliferation, as measured by ^{3}H-thymidine incorporation, was completely (but reversibly) inhibited. Leibovich and Polverini (1984) also observed that protamine could inhibit endothelial cell proliferation, adding the salient point that there were significant differences in the response of different cell types to this inhibitory action, neoplastic cells being the most sensitive. Whether or not this increased sensitivity of neoplastic cells extended to tumor-associated endothelium remains to be determined, but clearly such differential sensitivity might well form the basis for selective anti-angiogenic therapy.

M. B. Grant *et al.* (1993a,b) have reported that somatostatin analogues, such as octreotide, inhibit the stimulatory effect of retinal endothelial cell proliferation by IGF-1 and bFGF. They demonstrate that the anti-angiogenic effect is due, in part, to inhibition of growth factor-induced stimulation of proteases (see Section 3.2.1).

3.4.2. *Thrombospondins*

Bagavandoss and Wilks (1990) reported on the effect of a TSP-like protein inhibitor of angiogenesis originally described by Bouck and colleagues (Rastinejad *et al.*, 1989; Good *et al.*, 1990; Bouck, 1990), who attributed the production of this inhibitor to loss of a tumor suppressor gene. Since endothelial cells both produce TSP and express a receptor for this molecule, the Bagavandoss and Wilks' experiments analyzed the response of endothelial cells from a variety of sources (corpus luteum, adrenal cortex, pulmonary artery, umbilical vein) to human platelet-derived TSP. TSP was able to inhibit completely the FGF-induced proliferation of these three types of endothelial cells (see also Taraboletti *et al.*, 1990). Most interestingly, TSP, under similar conditions, was actually stimulatory to smooth muscle cells and fibroblasts, suggesting that the inhibitory action of TSP was endothelial-cell specific. This selective effect on endothelial cells is of considerable importance, for it distinguishes TSP from most of the inhibitors of proliferation. How the anti-angiogenic effect of TSP can be reconciled with the surprising finding of DiPietro and Polverini (1993) that activated monocytes and macrophages, which are potent inducers of angiogenesis, secrete markedly higher amounts of TSP than their unstimulated and non-angiogenic counterparts is not clear. The simplest, but not necessarily correct, explanation would be that the increase in anti-angiogenic factors, such as TSP, are more than offset by various angiokines (e.g. IL-1). However, Nicosia and Tuszynski (1994) report that TSP when bound to matrix, actually promotes angiogenesis, presumably by stimulating secretion of angiokines by myoblasts.

Iruela-Arispe *et al.* (1991) suggest that in addition to inhibiting endothelial cell proliferation, TSP destabilizes endothelial cell focal contacts. They argue further that the pro-angiogenic properties of heparin may stem, in part, from inhibition by heparin of the binding of TSP to endothelial cells. It is almost certain that differences between TSP1, TSP2 and TSP3 will be reflected in their relative anti-angiogenic activities (cf. Bornstein, 1992). To what extent the anti-angiogenic activity of TSP is modulated by its complexing with TGFβ is not known, but since the complex is biologically active and stable, part of the anti-angiogenic effect of TSP may be due to its protection of TGFβ from extracellular inactivation (Murphy-Ullrich *et al.*, 1992).

3.4.3. *Phorbol Esters*

We have already discussed the proangiogenic effect of tumor promoters by their induction of collagenase and plasminogen activator. However, a most intriguing report by Doctrow and Folkman (1987) documents selectivity in the effect of phorbol diesters. Whereas these protein kinase C-activating compounds are normally mitogenic, they *inhibit* cell division of bovine adrenal cortex capillary endothelial cells. The authors propose that the anti-mitogenic effect is achieved by binding to receptors on capillary endothelial cells and thereby rendering these cells insensitive to endothelial cell mitogens. This paradoxical effect of phorbol esters is limited not only to endothelial cells, but

specifically to *capillary* endothelial cells. Since it is these cells that are principally responsible for angiogenesis, these experimental findings may point to a new and most promising approach to anti-angiogenesis. It is worth recalling that a similar paradox was seen in the study of chondrocyte-derived inhibitor, which was reported to have anti-collagenase activity (hence effective in disrupting basement membrane), yet also inhibit capillary endothelial cell migration and proliferation (Moses *et al.*, 1992). Kosaka *et al.* (1993) use their results obtained by PMA treatment of HUVE cells to present a sequence of regulatory events in which PMA is seen as an inducer of protein kinase C, which, in turn, leads to suppression of cyclin A gene transcription and the subsequent arrest of endothelial cell proliferation. A similar role for phorbol esters in the control of angiogenesis has been proposed by Tsopanoglou *et al.* (1993b), based on their analysis of angiogenic responses on the chick CAM.

3.4.4. *Retinoids*

Another group of agents with marked effects on endothelial cell proliferation are the retinoids. For example, Braunhut and Palomares (1991) found that both bovine adrenal cortical capillary and aortic endothelial cells were reversibly growth-inhibited by retinol, while pericytes were not affected. They ascribed the inhihitory effect to an effect of retinol on cell shape (cf. Folkman and Moscona, 1978; Ingber, 1990, 1991). This change in cell shape may well be due to the action of retinol on endothelial cell synthesis of matrlx molecules (Paige *et al.*, 1991).

Imcke *et al.* (1991) found a dose-dependent significant inhibition of proliferation of dermal microvascular endothelial cells treated with etretin, etretinate or isotretinoin, that, however, did not, abrogate cytokine-induced changes in cell surface antigen expression. This may prove important from a therapeutic standpoint, since one may be able to target newly-induced antigens on endothelial cells without the complication of a concurrent mitogenic response. The concept that vascular endothelial cell targeting can be achieved by directing cytotoxic drugs to endothelium-specific antigens already has been validated by Burrows and Thorpe (1993), who directed immunotoxins to cytokine-induced endothelial cell markers.

The possibility that not all angiogenic reactions are inhibited by the same retinoids was raised by Szmurlo *et al.* (1992), who distinguished between acitretin and etretinate, with only acitretin being effective in inhibiting tumor-induced angiogenesis. Yasuda *et al.* (1992) suggest that the anti-angiogenic effect of retinoic acid is based, in large part, on its destructive effect on lysosomes, causing them to release hydrolytic enzymes, which, in turn, mediate destruction of endocytosed, intracellular bFGF. The fact that pentamolar quantities of retinoids are sufficient to induce avascular zones on the CAM prompted Oikawa *et al.* (1989a) to suggest that their anti-angiogenic effect may be mediated by modulating the signal transduction pathway. Ishii *et al.* (1992) have suggested that retinoic acid counteracts the pro-angiogenic (pro-thrombotic) action of inflammatory cytokines such as TNFα. The mechanism by which vitamin D_3 analogues inhibit angiogenesis in the CAM is also not known, although it has been postulated that these analogues share similarities with other retinoids (Oikawa *et al.*, 1990c, 1993c).

The anti-proliferative effects of genistein, an isoflavonoid found in soy products, has been well documented (Fotsis *et al.*, 1993). Whether the anti-angiogenic action of genistein may not solely be due to its anti-proliferative action, inasmuch as it also alters protease and protease inhibitor synthesis, affects the morphology of cultured endothelial cells and prevents tube formation in collagen gels (Fotsis *et al.*, 1993).

3.4.5. *The TGFβs*

One of the most potent inhibitors of endothelial cell proliferation *in vitro* is TGFβ, shown by Baird and Durkin (1986), Frater-Schroeder *et al.* (1986, 1987) and Heimark *et al.* (1986) to inhibit the *in vitro* mitogenic response of both bovine adrenal cortex-derived capillary and bovine aortic endothelial cells to aFGF or bFGF. Since TGFβ is widely distributed, these investigators suggest that TGFβ may act at many different sites (e.g. kidney, retina, ovary) to regulate the proliferative activity of endothelial cells during tissue repair. At least some of the TGFβ effects may be

modulated by glutathione, which, depending on its concentration, may enhance or inhibit the action of TGFβ on cultured endothelial cells (White *et al.*, 1992).

Recently, activins have been reported to inhibit proliferation of all types of endothelial cells *in vitro*, including HUVE cells, large vessel endothelial cells and microvascular endothelial cells (McCarthy and Bicknell, 1993). It is of interest that activin-A can be induced by TGFβ, and, thus, may be viewed as augmenting the anti-angiogenic action of the TGFβs.

Merwin and colleagues (1991) further distinguish between the antiproliferative effects of TGFβ1 and TGFβ2, and describe disparate responses of bovine aorta-derived large vessel endothelial cells and rat epididymal fat pad-derived microvascular endothelial cells to TGFβ2 (but not TGFβ1). Their assumption that the differences are due to the origin and type, rather than species of origin, needs to be confirmed experimentally. In this context, it is significant that rat cardiac microvascular endothelial cells can be induced to produce principally TGFβ2 when cultured in combination with heart myocytes (Nishida *et al.*, 1993).

TGFβ has also been identified in extracts from aqueous and vitreous humor, and the anti-angiogenic activity of these extracts was abrogated dramatically by pretreatment with antibodies to TGFβ (Antonelli-Orlidge *et al.*, 1989; Eisenstein and Grant-Bertacchini, 1991). Brem *et al.* (1976, 1977) had reported earlier on anti-angiogenic activity in the vitreous as measured in the corneal assay, while Okamoto *et al.* (1990) had shown that aqueous humor contains inhibitors of angiogenesis as measured both in the CAM assay and by inhibition of pulmonary endothelial cell proliferation *in vitro*, but reported that this inhibitory activity was lost in diabetic rabbits. It will be interesting to determine whether this loss is correlated with a reduced presence of TGFβ or whether other inhibitors are present in the anterior chamber of the eye. TGFβ is not the only inhibitor in the vitreous (Taylor and Weiss, 1985; cf. Brem *et al.*, 1976; Raymond and Jacobson, 1982; Lutty *et al.*, 1983, 1985), and the anti-collagenase activity, in particular (Taylor and Weiss, 1985), may be important in regulating periretinal angiogenesis. Inhibitory activity in the vitreous is increased following laser photocoagulation, and this activity is likely to represent an increase in a number of serum factors, including, but not limited to, TGFβ (Singh *et al.*, 1990). Extracts from human and bovine lenses also contained inhibitors of endothelial cell proliferation, but they have not been further characterized (Williams *et al.*, 1984).

The complex nature of regulating ocular neovascularization was well expressed by Glaser (1990): **"Each factor by itself is probably relatively meaningless, just as an individual number is in a combination used to open a lock. However several factors occurring in a specific combination can unlock neovascularization."** Glaser talks of growth factors as "extracellular modulating factors" that work together to induce or inhibit neovascularization.

Flaumenhaft *et al.* (1992) provide convincing evidence that the role of TGFβ is an extremely complex one, in which bFGF-induced uPA production leads to TGFβ activation, setting into motion a feedback loop that results in reduced endothelial cell proliferation. Additionally, Heimark *et al.* (1986) document that TGFβ inhibits not only cell proliferation, but cell movement in response to *in vitro* "wounding" of endothelial cells. This latter finding would suggest that antibodies to TGFβ would inhibit wound healing *in vivo*, and Shah *et al.* (1992) reported that neutralizing antibody to TGFβ reduced blood vessel formation associated with the healing of dermal wounds. Yet, TGFβ stimulates macrophages to produce uPA (Falcone *et al.*, 1993), an effect that is likely to augment rather than inhibit angiogenesis.

These studies point to two serious problems in interpretation: (1) TGFβ inhibits proliferation of many different cell types, including pericytes, fibroblasts and endothelial cells. This makes it difficult to evaluate how it might exert a selective effect on angiogenesis. (2) Whereas TGFβ inhibits endothelial cell proliferation *in vitro*, it is actually angiogenic *in vivo* (Roberts *et al.*, 1986). **This fact again illustrates one of the significant problems of utilizing *in vitro* correlates of angiogenesis: the question as to the extent to which we can rely on *in vitro* tests to determine efficacy of *in vivo* drug treatments.**

3.4.6. *Tumor Necrosis Factor, Interferons, Interleukins and Other Cytokines*

As mentioned in Sections 3.2.1 and 3.3.11, TNF has been shown to have paradoxical effects *in vivo*, including both induction and inhibition of basement membrane formation, and also to have

varying indirect effects on endothelial cell migration and proliferation (Sato *et al.*, 1986, 1987; Mano-Hirano *et al.*, 1987; Schweigerer *et al.*, 1987). Fajardo and colleagues (1992) have analyzed the effect of TNFα (TNF; cachectin) on angiogenesis *in vivo*, using their quantitative sponge implant (disc angiogenesis) assay. This assay measures the penetration of new blood vessels into a polyvinyl alcohol sponge, a reaction that requires both cell migration and cell proliferation. In their study of TNF, they correlated the growth of blood vessels into the sponge with ^{3}H-thymidine incorporation, thus providing data on TNF effects on the proliferative phase of angiogenesis. Their finding of a dose-dependent, dual response, consisting of *stimulation* of proliferation at low concentrations and *inhibition* of proliferation at high concentrations, is provocative, and raises the cautionary flag that dose–response data are essential in assigning an inhibitory role to a specific agent (see also Hicks *et al.*, 1989; Saegusa *et al.*, 1990; Ito *et al.*, 1990). It should be mentioned that, in these studies, the effect on proliferation was monitored by autoradiography, thereby excluding the possibility that the effect of TNF was entirely due to its demonstrated cytotoxic effects on endothelial cells (Schuger *et al.*, 1989). The sensitivity of endothelial cells to TNFα also appears to depend on their relative rate of proliferation, rapidly dividing cells being more sensitive to TNF inhibition than more slowly replicating ones (van de Wiel *et al.*, 1992). Not surprisingly, therefore, TNF was found to have an augmented growth-inhibiting effect when added to endothelial cells that had been permitted to "age" *in vitro* (Shimada *et al.*, 1990). The effect of TNFα was also enhanced by flavone acetic acid (Lin *et al.*, 1993). Roby and Terranova (1990) have isolated an endothelial cell-specific inhibitor from the ovary, which, they believe, on the basis of preliminary molecular analysis and by its lack of heparin affinity, to be identical or similar to TNFα.

Additional complexity in studying TNF effects was shown by Norioka *et al.* (1992), who were able to abrogate endothelial proliferation *in vitro* with *low* (non-cytotoxic; cf. Schuger *et al.*, 1989) doses of TNFα administered in combination with IL-1 and IFN-γ. It is significant that while IFN-γ by itself is anti-proliferative at high doses (Friesel *et al.*, 1987; Hicks *et al.*, 1989; Sato, N. *et al.*, 1990), apparently by reducing the number of binding sites for growth factors expressed on endothelial cells, the synergistic action of IFN-γ is exerted at concentrations well below those required for direct IFN-γ-induced inhibition of endothelial cell proliferation. Saegusa *et al.* (1990) found that *low* doses of IFN-γ caused a modest increase in proliferation of HUVE cells, whereas IL-1 was inhibitory (see also Norioka *et al.*, 1987). The reason for the opposite results of Saegusa *et al.* (1990) and Norioka *et al.* (1992) is likely to be due to differences in the type of endothelial cells studied, although species differences may also play a role in the response to cytokines. A synergism between IFN-γ and a lipid-A subunit analog has also been reported (Saiki *et al.*, 1992) and ascribed to the observation that IFN-γ induces lipid A, which, in turn, induces an increased production of TNFα. Contradictory results have also been obtained when various cytokines such as IL-1, IL-2 and IFN-γ were tested individually for their effect on proliferation (cf. Holzinger *et al.*, 1993), contradictions that cannot be resolved easily, especially since they mimic contradictions in results obtained when these cytokines were tested *in vivo*, as well as *in vitro*. There is a vast literature on cytokine induction and inhibition, which goes beyond the scope of our review. For example, since bradykinin augments the efficacy of IL-1 in inducing angiogenesis, inhibitors of bradykinin, as well as antagonists of IL-1 receptor, will manifest anti-angiogenic properties (Hu and Fan, 1993; Fan *et al.*, 1993). These authors also found that blocking of the NK_1 receptor inhibited angiogenesis, probably due to its blocking of substance P, a co-factor for IL-1-induced angiogenesis. Similarly, interactions have been shown between various ILs and inhibitors of cyclooxygenases (Delomenie *et al.*, 1993), in whose studies opposing effects were seen, depending on which of several cytokines (IL-1α, IL-1β, TNFα, TNFβ, TGFβ or monocyte-derived-endothelial cell inhibitor) was added in the proliferation assay, and whether other stimulators of angiogenesis, such as thrombin, were included in the test media. The anti-angiogenic effect of nicotinamide has been interpreted as resulting from the ability of nicotinamide to inhibit IFN-γ, rather than by a direct effect on endothelial cells (Hiromatsu *et al.*, 1991).

Another cytokine with profound effects on endothelial cells is leukemia inhibitory factor (Ferrara *et al.*, 1992). It is particularly interesting that these investigators found that leukemia inhibitory factor inhibits the proliferation of aortic endothelial cells, but fails to inhibit capillary endothelial cells. In contrast, the experiments of Miller *et al.* (1993), in which IFN-α was administered systemically, showed that newly arising vessels were sensitive to this cytokine, whereas established

vessels were not affected. Other examples of selective effects of anti-angiogenic agents on specific types of endothelial cells have already been cited. **Clearly, the question of endothelial cell heterogeneity should not be ignored in evaluating agents that inhibit angiogenesis** (cf. Alby and Auerbach, 1984; Auerbach *et al.*, 1985, 1987; Gumkowski *et al.*, 1987; Auerbach, 1991).

An interesting additional factor, "macrophage-derived endothelial cell inhibitor", was found by Besner and Klagsbrun (1991) to inhibit both base levels and FGF-induced proliferation of adrenal capillary endothelial cells *in vitro*. This inhibitor was distinct from other macrophage-associated inhibitors. Because of its strong binding affinity for heparin, its action may lie in competitive inhibition of heparin-binding growth factors.

The multiplicity of cytokine interactions, the selectivity of their target and the complex manner by which cytokine production is regulated frustrates any effort to formulate meaningful generalizations.

3.4.7. *Steroids and Heparin*

The effects of various steroids on endothelial cell proliferation have been described in an extensive series of studies by Folkman and colleagues (Folkman *et al.*, 1983; Folkman, 1985c, 1986; Crum *et al.*, 1985; Folkman and Ingber, 1987, 1989; Li *et al.*, 1991) as well as by many other investigators (Tanaka *et al.*, 1986; Cariou *et al.*, 1988; Sakamoto *et al.*, 1987; Sakamoto and Tanaka, 1988; Teicher *et al.*, 1992; Lee, K. *et al.*, 1987, 1990; Harada *et al.*, 1992; reviewed by Wilks, 1992). In particular, tetrahydro S and hydrocortisone have proven effective *in vitro*, as well as *in vivo* (Stokes *et al.*, 1990). A marked increase in efficacy of these steroids was seen when they were combined with heparin (Crum *et al.*, 1985; Cariou *et al.*, 1988; Sakamoto *et al.*, 1987; Folkman *et al.*, 1989) or heparin substitutes, such as maltose tetrapalmitate (Madarnas *et al.*, 1989). In fact, β-cyclodextrin tetradecasulfate, a highly potent synthetic heparin substitute, was *stimulatory* when administered singly, but markedly *inhibitory* when administered concurrently with hydrocortisone (Folkman *et al.*, 1989). Skubitz and Ehresmann (1992), however, showed that β-cyclodextrin inhibited ecto-protein kinase-mediated bFGF phosphorylation equally in the presence or absence of hydrocortisone. It is interesting, therefore, that tetrahydro S inhibited tumor-induced new blood vessel formation *in vivo* equally in the absence and presence of heparin, as measured in the alginate pellet system of Plunkett and colleagues (Robertson *et al.*, 1991; Downs *et al.*, 1992), and that the effect of cortisone on prostaglandin E_1-induced angiogenesis in the cornea was also not augmented by heparin (Ziche *et al.*, 1985). Unlike the angiostatic corticosteroids, estrogen metabolites such as 2-methoxyoestradiol were found to act independent of heparin, a finding that may be of considerable practical importance inasmuch as the concomitant administration of heparin may be avoided in the development of anti-angiogenic therapeutic protocols (Fotsis *et al.*, 1994). The observation that 2-methoxyoestradiol is effective in inhibiting the proliferation of bFGF-stimulated endothelial cells, but not of quiescent ones, is also noteworthy. The efficacy of the heparin–steroid conjugate generated by Thorpe *et al.* (1993) has been discussed earlier. The ability of the non-coagulant heparin fragment/hydrocortisone conjugate to inhibit murine pulmonary endothelial cell proliferation was several orders of magnitude greater than that of the mixture of heparin and hydrocortisone.

Synergistic or additive effects were also observed when steroids were coadministered with DS4152, a bacterially derived sulfated polysaccharide complex (discussed in Section 3.4.13) (Inoue *et al.*, 1988; Tanaka *et al.*, 1989, 1991). Folkman and Shing (1992) have provided a thoughtful review of the control of angiogenesis by heparin and other sulfated polysaccharides.

Inhibitors of arylsulfatases were found to potentiate the steroid effect, especially when heparin concentrations were suboptimal (Chen *et al.*, 1988). Several studies, however, indicate that the primary action of most of the angiostatic steroids is exerted on basement membrane synthesis (Folkman and Ingber, 1987) and that the anti-proliferative effect may be secondary to this action (Ingber *et al.*, 1986; Form *et al.*, 1986).

3.4.8. *Suramin*

Suramin, a polysulfonated urea that has been demonstrated to be anti-angiogenic, is one of many agents that may affect the angiogenic process at several points (Stein, 1993). Suramin binds to

growth factors such as TGFβ or EGF, thus reducing cell division indirectly (see also Danesi *et al.*, 1993). It may augment the efficacy of angiostatic steroids manifested by cytostasis (Gagliardi *et al.*, 1992) and, significantly, by inhibition of angiogenesis on the CAM (Wilks *et al.*, 1991; Danesi *et al.*, 1993). At the same time, it causes a change in cell shape and in the expression of cell-surface adhesion molecules (Fantini *et al.*, 1990), both of which markedly influence the capacity of endothelial cells to respond to growth factors. Suramin has also been shown to have a striking effect on cells transfected with a bFGF construct that includes a signal sequence to produce an autocrine FGF-producing cell with increased proliferation rate. Addition of suramin inhibits this autocrine transformation by down-regulating the FGF receptor, thus returning the transfectant to its normal basal level of cell replication (Yayon and Klagsbrun, 1990). It is likely that the suggested efficacy of suramin as an anticancer drug (Zaniboni, 1990; Gil *et al.*, 1993) is due, in part, to its effect on growth factor receptors on transformed cells. Theobromine, which like suramin is a purinerginic receptor antagonist with anti-tumor properties, has also been found to be mildly anti-angiogenic (Gil *et al.*, 1993). The ability of suramin to block the binding of vasculotropin to its receptor has been mentioned already (Plouet and Moukadari, 1990).

As mentioned in Section 3.4.1, M. B. Grant *et al.* (1993b) recently reported that insulin-like growth factor (IGF) was angiogenic when placed in the rabbit cornea. Since suramin can inhibit IGF-I (Pollak and Richard, 1990), part of the anti-angiogenic effect of suramin may be through its suppression of IGF-I action. On the other hand, the effect of suramin on IGF-I and IGF-II is complex (Kraft *et al.*, 1993), for the reason that at least part of its action on cells is complementary to the IGFs.

3.4.9. α_2-*Macroglobulin*

When VEGF binds to α_2-macroglobulin, its receptor binding ability is inhibited (Soker *et al.*, 1993). A most interesting finding in their study was that heparin, as well as heparan sulfate, was able to inhibit this complexing of VEGF, providing a different view of how heparin might act indirectly to augment growth factor efficacy, i.e. preventing growth factor inactivation.

3.4.10. *Antibodies to Growth Factors*

As a general rule, neutralizing antibodies to growth factors will inhibit the action of those growth factors. For example, we have already mentioned that antibodies to bFGF will inhibit FGF-induced angiogenic responses (Sakaguchi *et al.*, 1988; Matsuzaki *et al.*, 1989; Reilly *et al.*, 1989; Gross *et al.*, 1992). Antibodies to bFGF also blocked angiogenesis induced by human glioma cell lines (Abe *et al.*, 1993). Antibodies to peptides of VEGF were able to block VEGF-induced endothelial cell proliferation (Sioussat *et al.*, 1993). The effect of antibodies to VEGF thus parallels the effect elicited by the administration of soluble receptors to VEGF (Kendall and Thomas, 1993). A particularly significant observation, however, was made by Kim *et al.* (1993). These investigators reported that neutralizing antibodies to VEGF were able to inhibit the growth of human rhabdomyosarcoma, glioblastoma multiforme and leiomyosarcoma cells injected into athymic mice, even though the antibody had no effect on the growth of these tumor cells *in vitro*. These tumors all expressed mRNA for VEGF, supporting the argument that anti-VEGF is effective by virtue of its inhibition of VEGF-induced angiogenesis. The recent report by Millauer *et al.* (1994), which documents the ability of a dominant-negative Flk-1 mutant to block glioblastoma growth, provides further support for the importance of blocking VEGF as an angiogenesis-inducing agent, inasmuch as Flk-1 functions as the major receptor for VEGF.

Hepatocyte growth factor (scatter factor), like FGF, is complex in its action: it promotes endothelial cell migration, proliferation and tube formation *in vitro*; it promotes the production of urokinase; and it induces blood vessel formation *in vivo* (Grant, D. S. *et al.*, 1993). The angiogenic activity of scatter factor, however, can be completely abolished by anti-scatter factor antibodies.

3.4.11. *Anti-Angiogenic Peptides*

There are numerous peptide fragments that may inhibit endothelial cell proliferation. For example, the 16 kDa fragment of prolactin is inhibitory to both the basal level (standard cell culture medium containing 10% fetal bovine serum) and FGF-stimulated proliferation of bovine brain or adrenal capillary endothelial cells (Ferrara *et al.*, 1991; Clapp *et al.*, 1993). This finding is of special interest because it contrasts with the mitogenic effect of the same fragment on epithelial cells. Similarly, heparin-binding peptide fragments from fibronectin inhibit endothelial cell proliferation (Homandberg *et al.*, 1986), as do selected peptides of TSP (Tosma *et al.*, 1993; Vogel *et al.*, 1993).

Itoh *et al.* (1992) reported that atrial natriuretic polypeptide markedly inhibited cell proliferation of bovine aortic endothelial cells, and this effect was mimicked by cyclic GMP, suggesting that this polypeptide might exert its effect through the involvement of guanylate cyclase-dependent mechanisms.

With respect to peptide-induced inhibition, results obtained with PF4 are particularly informative. PF4, discussed in Section 3.3.6 because of its effect on cell migration, had been assumed to be anti-angiogenic because of its affinity for heparin. As expected, addition of heparin to PF4 abolished its anti-angiogenic activity (Maione *et al.*, 1990). However, Maione *et al.* (1991) have now demonstrated that a non-heparin-binding analog of PF4, rPF4-241, is still angiostatic, and they correlate this activity with the ability of the analog to inhibit endothelial cell proliferation *in vitro* (cf. also Sharpe *et al.*, 1990). These studies show, as do the studies of laminin and TSP fragments, that **the effects on angiogenesis elicited by an intact molecule may be quite different from the effects of individual peptides generated from these molecules either experimentally or during the course of normal degradation.**

3.4.12. *Retina-Derived Inhibitors*

The report of McIntosh *et al.* (1989) that a crude extract of the retina in combination with adult serum causes a marked reduction in proliferation of retinal endothelial cells is intriguing because the effect is selective to the extent that retinal pericytes are not affected. The study leaves unanswered the question of whether the effect is restricted to *retinal* endothelial cells or whether it affects endothelial cells from other organs. It may be that the activity in their extract is identical to the inhibitor that Glaser *et al.* (1985, 1988; cf. Karasawa and Okisaka, 1990) identified in supernatants obtained from cultured retinal pigment epithelial cells.

3.4.13. *Antibiotics*

Akselband *et al.* (1991) analyzed the anti-proliferative effect of rapamycin, an antibiotic inhibitor of T-cell proliferation, on bovine aortic endothelial cells, HUVE cells and murine 3T3 fibroblasts. All of these cells show strong proliferative responses to bFGF. When rapamycin was added to bFGF, proliferation was blocked almost completely. From the standpoint of drug development, moreover, it was interesting that these results contrasted with related inhibitors, such as FK 506, a structurally-related macrolide, the latter having no effect on FGF-stimulated cell proliferation. Oikawa *et al.* (1991) described strong anti-proliferative activity of eponemycin on bovine carotid endothelial cells grown in collagen gels, correlating with its inhibitory effects on angiogenesis in the chick CAM assay. It should be recalled that these investigators also demonstrated an anti-migration effect of this antibiotic (Section 3.3.17). Another antibiotic studied by Oikawa and his colleagues (1992a) was the spermidine moiety-containing compound 15-deoxyspergualin. One of the surprising findings of their study of this antibiotic was that no anti-proliferative effect could be observed under standard monolayer *in vitro* culture conditions. However, when cells were cultured in a three-dimensional collagen gel, this antibiotic proved to be an effective inhibitor of endothelial cell proliferation.

TAN-1120, a baumycin-group anthracycline, was found to be a potent selective inhibitor of endothelial cell proliferation, and prevented bFGF-induced neovascularization of the rat cornea (Nozaki *et al.*, 1993). TAN-1120 was found to be four orders of magnitude more inhibitory than either doxorubicin or daunomycin in the CAM assay, yet it had no effect on three-dimensional tube formation on Matrigel.

D-Penicillamine is another antibiotic with anti-proliferative activity, although its effect requires copper as a cofactor (Matsubara *et al.*, 1989; Brem *et al.*, 1990). Whether the requirement for copper is due to the ability of that metal to cause dimers with aFGF (Engleka and Maciag, 1992) is not known. It is worth recalling earlier studies by Francois *et al.* (1973), which attributed penicillamine effects to its ability to reduce collagenase activity.

Another antibiotic with potent anti-proliferative effects on endothelial cells is fumagillin, as well as its more potent synthetic analogue AGM-1470 (TNP-470) (Ingber *et al.*, 1990; Kusaka *et al.*, 1991; Marui *et al.*, 1992; Kamei *et al.*, 1993; Yanase *et al.*, 1993; Nguyen *et al.*, 1994). Ingber and colleagues classify fumagillin and AGM-1470 as "angioinhibins", emphasizing that they are neither steroid, polysaccharide, retinoid nor peptide structures (Ingber, 1992; Ingber *et al.*, 1990). Recently, it was shown that AGM-1470 prevents the entry of endothelial cells into the cell cycle by blocking the transition from G_0–G_1 (Antoine *et al.*, 1994). That AGM-1470 may have a more complex mode of interfering with angiogenesis, however, is suggested by the observation that AGM-1470 can cause a prolonged anti-inflammatory effect (>2 months) on collagen-induced arthritis (Peacock *et al.*, 1992), although these investigators propose that it is the initial anti-proliferative action of the antibiotic that prevents the initial angiogenic component of the disease. Whatever the mechanism or mechanisms, it is important to note that this compound has been shown to reduce the metastatic spread of several human lung tumors transplanted into nu/nu athymic mice, and that this effect was correlated with a reduction in the number of capillaries around the original tumor implant (Yanase *et al.*, 1993). The anti-tumor effect of AGM-1470 on schwannomas and neurofibromas was also correlated with a reduction in tumor-associated blood vessels (Takamiya *et al.*, 1993). Brem and Folkman (1993) reported anti-tumor effects of AGM-1470 on murine Lewis Lung carcinomas, Colon 38 carcinoma and Fibrosarcoma 105. AGM-11470 was also able to inhibit the growth and number of metastatic foci of B16 melanoma cells, as well as of M5076 reticulum sarcoma cells (Yamaoka *et al.*, 1993b) and of hormone-independent breast and prostate carcinoma cell lines (Yamaoka *et al.*, 1993a). Additive inhibitory effects on 3LL lung tumor growth were observed when AGM-1470 was combined with IFN-α/β, as determined both by measurement of the primary tumor mass and by determining the number of pulmonary metastatic foci (Brem *et al.*, 1993). The fact that coadministration of doxorubicin with AGM-1470 augmented the anti-tumor effect is particularly interesting in view of the fact that doxorubicin by itself was found to be only weakly anti-angiogenic (cf. Nozaki *et al.*, 1993).

Anti-proliferative effects on HUVE cells, as well as anti-angiogenic effects on the CAM, were also shown for compound FR-111142, another fungal antibiotic, which was isolated from strain F-2015 of *Scolecobasium arenarium* (Otsuka *et al.*, 1992a). WF-16775A_1 and A_2, isolated from *Chaetasbolisia erysiphoides*, were also found to be anti-angiogenic in both the endothelial cell proliferation and CAM assays (Otsuka *et al.*, 1992b).

Tanaka and colleagues have described yet another microbial angiostatic agent, SP-PG (or its most active component, DS-4152), a sulfated polysaccharide–peptidoglycan complex produced by an *Arthobacter* species (Inoue *et al.*, 1988; Tanaka *et al.*, 1989). DS-4152 inhibits tumor-associated vascularization and has shown efficacy in reducing the angiogenic response to Kaposi's sarcoma cells on the CAM or in nude mice (Nakamura *et al.*, 1992). It is worth noting that SP-PG or DS-4152 action is markedly enhanced by concomitant administration of tetrahydrocortisone and other steroids (Inoue *et al.*, 1988; Nakamura *et al.*, 1992; Tanaka *et al.*, 1991). A similar compound, purified from the mycelia of *Coriolus versicolor*, was also reported as reducing tumor growth, and its action was also ascribed, at least in part, to its suppression of tumor vascularization (Kumar *et al.*, 1992).

The anti-angiogenic effect of tetracyclines generally has been attributed to their interference with collagenase activity. However, Guerin *et al.* (1992) reported that minocycline showed a cell-type selective effect on endothelial cells, reducing their growth rate by 80% when minocycline was added in micromolar concentrations to retina-derived microvascular endothelial cells.

3.4.14. *Glycosaminoglycans*

West and colleagues have carried out an extensive set of studies on the effects of hyaluronan on angiogenesis (West *et al.*, 1985; West and Kumar, 1989, 1991). Following up their finding that high

molecular mass ($>10^6$ kDa) hyaluronan was found to inhibit vessel formation during wound healing, they assessed the effect of hyaluronan on proliferation of bovine aortic endothelial cells *in vitro*. Interestingly, they found that high molecular weight hyaluronan was an effective inhibitor of endothelial cell proliferation, but that hyaluronan oligosaccharides (3–16 disaccharides in length) stimulated proliferation of these cells (West and Kumar, 1991; Sattar *et al.*, 1992). The latter finding correlates with the demonstration that low molecular mass hyaluronan (2–10 kDa) stimulated angiogenesis both in the chick CAM and in a rat wound healing model (results cited in West and Kumar, 1991).

3.4.15. *SPARC*

As discussed in Section 3.3.9, Sage and colleagues (Funk and Sage, 1991; Sage, 1992; Hasselaar and Sage, 1992) reported on multiple anti-angiogenic properties of a secreted protein (SPARC). Extending their initial demonstration that SPARC prevents entry of aortic endothelial cells into S-phase of the cell cycle, they showed that SPARC inhibited ^{3}H-thymidine incorporation into endothelial cells both in the absence or presence of bFGF (in contrast to the requisite role of bFGF in SPARC effects on cell movement), but that SPARC did not inhihit proliferation when endothelial cells were plated on fibronectin or gelatin in serum-free medium. Even further complexity in assigning a role to SPARC in angiogenesis was introduced by Lane *et al.* (1992), who have shown that SPARC also regulates the synthesis of several key molecules critical to the angiogenic process: SPARC decreases fibronectin and TSP-1, while it increases PAI-1.

3.4.16. *Other Pharmacological Agents*

Chloroquine, an anti-malarial drug, has paradoxic effects with respect to angiogenesis in that it promotes tissue plasminogen activator production and potentiates bFGF-induced cell migration, but it can completely inhibit ^{3}H-thymidine incorporation into human microvascular endothelial cells *in vitro* (Inyang *et al.*, 1990). The anti-angiogenic action of magnosalin, a compound extracted from magnolias, used as an anti-inflammatory agent, has also been ascribed to its ability to inhibit endothelial cell proliferation (Kimura *et al.*, 1990). Other drugs with antiproliferative effects include sulfapyridine (Madhok *et al.*, 1991), several opioids (Pasi *et al.*, 1991) and various gold compounds (Matsubura and Ziff, 1987). Dimethyl sulfoxide is able to inhibit endothelial cell proliferation in a reversible manner (Layman, 1987), endothelial cells being more resistant to dimethyl sulfoxide than smooth muscle cells. This finding may be of particular interest to investigators attempting to obtain pure cultures of endothelial cells.

One cannot overemphasize the need for circumspection in evaluating *in vitro* correlates of angiogenesis such as endothelial cell proliferation. This is true not only with positive results, but with negative ones as well. For example, eicosapentaenoic acid, which had been found not to affect endothelial cell proliferation under standard culture conditions, in fact did inhibit endothelial cell proliferation when the endothelial cells were cultured between two collagen gel layers (Kanayasu *et al.*, 1991). The assay used in these studies more closely reflects the situation in which endothelial cells find themselves *in vivo* than does the standard microwell protocol usually used to assess endothelial cell proliferation *in vitro*. This illustrates an important principle: **Care must be taken to select, wherever possible, those *in vitro* protocols that most closely approximate the conditions found *in vivo*.**

3.5. INHIBITORS OF THREE-DIMENSIONAL ORGANIZATION AND ESTABLISHMENT OF PATENCY OF NEW BLOOD VESSELS

3.5.1. *The TGFβs*

The extensive studies by Madri and colleagues on the effects of TGFβ underscore the complexity of the process of vascular restructuring. Many of the effects observed *in vitro* depend on the properties of the extracellular matrix (Madri *et al.*, 1988), and it is likely that differences in matrix composition *in vivo* would lead to highly significant differences in the vascular response to

growth-regulating molecules. Madri *et al.* (1992) point out that TGFβ1, for example, affects proliferation, migration, extracellular matrix synthesis, protease inhibitor production and tube formation. While TGFβ1 and TGFβ3 affect proliferation of endothelial cells, TGFβ2 does not. On the other hand, TGFβ2 is the most potent inhibitor of tube formation. These authors further emphasize that different endothelial cells may respond differently to the TGFβs, i.e. there are marked differences between the response of microvascular and that of large vessel endothelial cells to these growth-regulating factors. Okamura *et al.* (1992) further demonstrated that TGFβ could inhibit TGFα-induced tube formation of omentum-derived microvascular endothelial cells plated on type I collagen gels.

An extensive set of experiments by Pepper and colleagues (1993) was carried out to determine the effect of TGFβ on bFGF- and VEGF-induced angiogenesis, as measured by the ability of capillary endothelial cells to form tubes in three-dimensional collagen gels. Their finding that TGFβ1 potentiates bFGF- and VEGF-induced invasion and tube formation when added at low doses, but inhibits the same process at high doses, may provide an explanation for the paradoxical results obtained in different laboratories utilizing various test systems and TGFβ concentrations.

3.5.2. *Interferons*

Among the cytokines, IFN-γ has been shown to inhibit tube formation of HUVE cells cultured on Matrigel (Maheshwari *et al.*, 1991). These investigators contrast this finding with their observations on IFN-α, which they report to be a promoter of tube formation under the same experimental conditions. It should be recalled (Section 3.3.2) that IFN-α has demonstrated paradoxical anti-angiogenic properties when tested *in vivo* (Sidky and Borden, 1987; Orchard *et al.*, 1989; Ezekowitz *et al.*, 1992).

3.5.3. *Fatty Acids*

Eicosapentaenoic acid, aside from being able to inhibit endothelial cell proliferation (Section 3.4.16) was also found to be a potent inhibitor of tube formation *in vitro*, contrasting with the effect of arachidonic acid, which was stimulatory (Kanayasu *et al.*, 1991). It is worth noting that the effect of eicosapentaenoic acid is diametrically opposed to the effect of TGFβ, which inhibits proliferation in monolayer cultures of endothelial cells, but promotes endothelial cell reorganization in three-dimensional culture (Madri *et al.*, 1988).

α-Guaiaconic acid, another inhibitor of arachidonic acid metabolism, was also inhibitory to *in vitro* tube formation, as shown by the failure of omentum-derived microvascular endothelial cells to form three-dimensional structures when plated on Matrigel (Ito *et al.*, 1993).

3.5.4. *Oxazolones*

Wright *et al.* (1992) tested the effect of two different oxazolones on the ability of umbilical vein endothelial cells to form tubes on Matrigel, as well as their ability to inhibit angiogenesis in the chick CAM. MD 27032 (4-propyl-5-(4-pyridinyl-2(3H)-oxazolone) prevented tube formation on Matrigel and was a potent inhibitor of angiogenesis in the CAM assay. In addition, this compound markedly reduced the adhesion of HUVE cells to laminin, fibronectin and fibrinogen. The effect was attributed to the ability of MD 27032 to inhibit protein kinase C, although other targets of action were not excluded.

3.5.5. *Inhibitors of Basement Membrane Biosynthesis*

In view of the fact that basement membrane formation is essential for three-dimensional vessel formation, agents that interfere with basement membrane biosynthesis will have anti-angiogenic properties (Maragoudakis *et al.*, 1993). Maragoudakis and colleagues have surveyed a large number of drugs that interfere with the formation of basement membranes by inhibiting collagen synthesis and have demonstrated that such drugs inhibit angiogenesis in the chick CAM assay (Maragoudakis *et al.*, 1988, 1989, 1990). Similarly, lymphotoxin and IFN-γ have been shown to inhibit

collagen formation, and Tsuruoka *et al.* (1988) point out that cytokine effects may be mediated not only by directly interfering with endothelial cell proliferation but also by preventing basement membrane biosynthesis.

Furumichi *et al.* (1992) elicited tube formation by HUVE cells with endothelial cell growth factor (brain-derived), then demonstrated that cyclic adenosine monophosphate and two derivatives of cyclic AMP could inhibit this reaction, whereas cyclic GMP had no effect on growth factor-induced tube formation. Their results complement those reported by Itoh *et al.* (1992), who observed a profound effect of 8-bromo cyclic GMP on endothelial cell proliferation.

One of the most faithful models for three-dimensional vessel formation is the aortic ring model developed by Nicosia and colleagues (Nicosia *et al.*, 1982; Nicosia and Madri, 1987). Nicosia *et al.* (1991) demonstrated that collagen-dependent vessel formation could be inhibited by *cis*-hydroxyproline, an inhibitor of collagen production. Additional support for the specificity of this inhibition comes from studies carried out with other collagen inhibitors (Nicosia *et al.*, 1991; Ingber and Folkman, 1988).

3.5.6. *Inhibitors of Cell Adhesion Molecules*

Attachment to laminin is crucial to three-dimensional reorganization of endothelial cells, and this finding was clearly demonstrated by studies of Kleinman and colleagues (Kubota *et al.*, 1988; Grant *et al.*, 1989, 1990; Kleinman *et al.*, 1993), who used soluble YSIGR-containing peptides to inhibit tube formation *in vitro*. The importance of endothelial cell attachment to matrix molecules was underscored by Nicosia and Bonanno (1991; Nicosia *et al.*, 1993), who suggested that in addition to blocking cell movement (discussed in Section 3.3.13), peptides that block integrin-mediated adhesion, such as the Arg–Gly–Asp (RGD)-containing peptide Gly–Arg–Gly–Asp–Ser (GRGDS) also inhibit tube formation in three-dimensional gel cultures. The effect of the RGD-containing peptide on angiogenin-induced cell adhesion has already been cited (Soncin, 1992). Previous reference has also been made to the complex action of SPARC, including its inhibition of fibronectin and TSP synthesis (Lane *et al.*, 1992). An intriguing finding of that study was that SPARC had no effect on endothelial cell lines that were unable to form tubes or cords under standard *in vitro* conditions.

Davis *et al.* (1993) studied the role of both vitronectin and fibronectin receptors in permitting endothelial cells plated in Matrigel. Polyclonal antibodies to either of these receptors were effective in blocking *in vitro* tube formation.

Angiogenesis induced by bFGF has been shown to lead to increased expression of $\alpha_v\beta_3$ integrins. The significance of this observation is underscored by the finding that antibodies to $\alpha_v\beta_3$ inhibit both bFGF-induced angiogenesis and tumor-induced angiogenesis as measured in the chick CAM assay system (Brooks *et al.*, 1994).

E-selectin, a cell adhesion molecule, binds to neutrophils by affinity to the lectin domains of cell surface-associated sialyl Lewis-X. This, in turn, increases binding of the neutrophils to activated endothelial cells, leading to endothelial cell tube formation *in vitro*. Antibodies to E-selectin were able to completely inhibit this response (Nguyen *et al.*, 1993). These investigators tested antibodies to related cell surface glycoconjugates (e.g. α-Lewis X, Lewis-B, Lewis A), but these did not inhibit tube formation. Thus, inhihition of E-selectin binding by interfering with its sialyl Lewis-X ligand has the potential to be a specific inhibitor of inflammation-associated angiogenesis.

3.5.7. *Other Inhibitors of Three-Dimensional Organization of Endothelial Cells*

Nicardipine, a calcium channel blocker, cited in Section 3.3.18 as inhibiting endothelial cell migration, proved to be effective in disrupting, but not completely inhibiting, the process of tubular structure formation of bovine aortic endothelial cells seeded between two layers of collagen (Kaneko *et al.*, 1992). Phosphokinase C inhibitors, such as calphostin C and staurosporine, also inhibited tube formation (Davis *et al.*, 1993).

An interesting recent report describes the anti-angiogenic effect of a chimeric toxin in which aFGF was fused to mutant forms of *Pseudomonas* exotoxin (aFGF-PE; Merwin *et al.*, 1992). Aside from a general cytotoxic effect not restricted to endothelial cells, aFGF-PE prevented

TGFβ-induced tube formation, as well as the migration of endothelial cells out of these cultures to spread as a monolayer. Why a chimeric compound generated with aFGF is effective is not clear, although it may be that FGF is an essential co-factor in permitting the three-dimensional organization of endothelial cells in matrix cultures. In this context, it is noteworthy that Sato *et al.* (1993b) found that a heparin-binding fragment of PF4 blocked the bFGF receptor on bovine aortic endothelial cells, and that this resulted in a reduction in their ability to form three-dimensional tubes in collagen gels.

A new three-dimensional *in vivo* assay was used by Passaniti *et al.* (1992) to assess the ability of various cytokines to inhibit the angiogenic response to aFGF. They found that IL-1β, IL-6, TGF-β and platelet-derived growth factor-BB all inhibited TGFα-induced three-dimensional vessel growth into a Matrigel plug.

Irsogladine, an anti-ulcer drug, was shown by Sato *et al.* (1993a) to inhibit tube formation by cultured omental microvascular endothelial cells, as well as to inhibit angiogenesis in a dorsal air sac model. Although irsogladine inhibited plasminogen activator production, these investigators were careful to point out that the drug may have other actions, including the disruption of gap-junctional communication. Among the retinoids, fenretinide was found to inhibit tubule formation in collagen gels (Pienta *et al.*, 1993). A proline analog, L-azetine-2-carboxylic acid, was inhibitory *in vivo*, where it prevented mast cell-mediated angiogenesis in a rat mesenteric window assay system. This was manifested by reduced vascular divarication and tortuosity (Norrby, 1993b). Cyclosporine, known best for its immunosuppressive properties, also prevented branching and tortuosity of blood vessels in this system, possibly by negating lymphokine secretion (cf. Sidky and Auerbach, 1975; Auerbach *et al.*, 1991b).

The 16 kDa fragment of prolactin, previously described (Section 3.4.11) as having an anti-proliferative effect *in vitro*, also inhibited three-dimensional restructuring of these cells plated on collagen gels. Since tube formation does not require proliferation this suggests that this peptide may affect angiogenesis at more than one place in the angiogenic process.

3.6. Physiological and Physical Interventions

3.6.1. *Cell–Cell Interactions*

Inhibition of angiogenesis by cell–cell contact is a major means of regulating the neovascular response. How different cells achieve this inhibition is not always clear. Even when an inhibitory substance can be isolated from a given tissue, it does not prove that this substance is necessarily the principal, or only, factor responsible for cell–cell-mediated inhibition of angiogenesis.

Several good examples of inhibition by cell–cell contact exist in ocular neovascularization (Glaser, 1989). One of the chief cell types with demonstrated anti-angiogenic properties is the pericyte (Orlidge and D'Amore, 1987; Miller *et al.*, 1986). Miller *et al.* ascribe pericyte activity to the ability of pericytes to surround the endothelial cells, binding them tightly and thus preventing leakiness (and concurrently reducing the accessibility of the endothelial cells to angiogenic factors). Coculture experiments (see Saunders and D'Amore, 1992) indicate that inhibition is also achieved through the production of TGFβ, shown experimentally by the loss of pericyte-mediated inhibition through the administration of antibodies to TGFβ (Antonelli-Orlidge *et al.*, 1989; Eisenstein and Grant-Bertacchini, 1991).

The efficacy of aldose reductase inhibitors in preventing retinal vascular pathology in diabetes has been attributed to their inhibition of aldose reductase-mediated destruction of the pericytes (Robison, 1988; Robison *et al.*, 1989a,b, 1990). The effect of aminoguanidine treatment, which reduces the accumulation of advanced glycosylation products on collagen and basement membrane, has also been attributed to its preventing the early loss of pericytes (Hammes *et al.*, 1991). A useful model for further study of endothelial–pericyte interactions is the subcutaneous disc assay in which Wakui *et al.* (1993) have been able to demonstrate time-dependent changes in cellular infiltrates, providing a strong correlation between pericyte influx and a decline in the number of endothelial cells.

Stromal–epithelial cell interactions are also involved in the regulation of neovascularization. Kaminska and Niederkorn (1993) carried out reciprocal corneal grafts between normal and athymic mice to determine the basis for the higher angiogenic activity in the athymic animals. They reported that the athymic mouse corneal epithelium produced increased angiogenic, as well as decreased anti-angiogenic factors in the epithelium, whereas stromal cells in both euthymic and athymic animals exerted anti-angiogenic effects.

Interaction between muscle cells and microvascular endothelial cells in the heart was shown by Nishida *et al.* (1993), who reported that endothelial cell proliferation was markedly reduced in cocultures of cardiac microvascular endothelial cells and ventricular myocytes. They ascribe this effect to increased production of TGFβ precursor mRNA, especially TGFβ2. Smooth muscle/endothelial cell interactions also have been reported to lead to angiogenesis inhibition (Laug, 1985), and this inhibition was attributed to the increase in PAI.

Cell interactions between astrocytes and endothelial cells were shown to be critical for neural microvascular differentiation. Using an *in vitro* assay system, Wolff *et al.* (1992) demonstrated that astrocyte–endothelial cell interactions are critical for microvascular differentiation into three-dimensional capillary-like structures. With this assay system, they were able to demonstrate the anti-angiogenic effect of dexamethasone, hydrocortisone and progesterone, whereas these same steroids did not block astroglial-independent capillary formation induced by Matrigel.

Even though in this review we have excluded the broad area of interactions between the endothelium and circulating blood leukocytes, we would be remiss if we were not to emphasize that these interactions are important in the control and regulation of angiogenesis. Similarly, we have not discussed specific interactions between tumor cells and endothelial cells, although it was these interactions that first directed attention to the question of angiogenesis and that even now represent the prime focus of anti-angiogenesis research.

3.6.2. *Blood Flow*

Blood flow is of fundamental importance in regulating the response of endothelial cells to angiogenic and anti-angiogenic agents (Jain, 1991; Simionescu and Simionescu, 1991). In addition, flow itself through its hydrodynamic effects influences endothelial cell shape and organization (Ingber, 1990). A discussion of the many factors which regulate blood flow as, for example, by vasodilation or vasoconstriction, by alterations in blood pressure or by induction or blocking of vascular shunts or channels is worthy of a review of its own, and will not be covered here.

3.6.3. *Photodynamic Therapy*

Although the bulk of anti-angiogenesis research has been directed at identifying biochemicals that could modulate inducers of angiogenesis by direct interaction with those inducers or by competing for their binding sites, it is as well to emphasize that physical intervention is another treatment modality. This is especially true in the eye, where photocoagulation or photodynamic therapy have been used effectively to inhibit angiogenesis and cause regression of neovascularization (Epstein *et al.*, 1987).

3.6.4. *Hyperthermia*

Another method of intervention is to use hyperthermia to inhibit angiogenesis. While the anti-tumor effect of hyperthermia has generally been ascribed to direct action on growing tumor cells, hyperthermia also has an inhibitory effect on the microvasculature (cf. Song, 1984). This effect has been investigated by Fajardo and colleagues (Fajardo *et al.*, 1988b; Kowalski *et al.*, 1992). Their studies showed that when polyvinyl alcohol foam discs containing epidermal growth factor are implanted into mice, the degree of vascular penetration is markedly reduced by local hyperthermia. They suggested that the effect of hyperthermia may be exerted by a combination of endothelial cell killing, inhibition of replication, inhibition of cell migration or by a combination of these mechanisms. They also caution that hyperthermia effects may differ for endothelial cells from different organ sites, for lymphatic versus blood capillaries and even within given capillary beds.

3.6.5. *Hypoxia*

In large part, a similar complexity is seen when endothelial cells are exposed to long-term reduced hypoxic conditions *in vitro* (Shreeniwas *et al.*, 1991). Both microvascular (bovine adrenal) and macrovascular (bovine aortic) endothelial cells failed to migrate into a "wounded" monolayer area. However, in addition to inhibiting cell movement, the cells at the wound edge also failed to proliferate, as measured by ^{3}H-thymidine incorporation. At the same time, hypoxia induced endothelial cell surface changes and altered vascular permeability. It should be kept in mind that hypoxia, as such, can hardly be considered an anti-angiogenic agent—indeed, hypoxia can *induce* angiogenesis in the developing chick CAM (Dusseau and Hutchins, 1988)—yet its effects on cell movement, proliferation, matrix interactions and susceptibility to growth-modulating effectors parallel many of the effects of pharmacological and cellular modulators of the neovascular process. We must caution, however, that although *in vitro* "wound healing" is considered a good model for *in vivo* cell migration, hypoxia *in vivo* appears to stimulate, rather than inhibit, angiogenesis, at least in the chick CAM system (Strick *et al.*, 1991).

4. FUTURE DIRECTIONS

Much new information is likely to come from genetic manipulation of cells and animals. For example, Huber *et al.* (1992) have introduced the human immunodeficiency virus *trans*-activator gene (*tat*) into HeLa cells and shown that such transfected cells produce fewer tumors in nude mice than do non-transfected cells. They ascribe this reduction in tumor growth to an anti-angiogenic effect of *tat*-gene expression and identify one polypeptide that is altered as a result of the introduced gene. However, is this peptide anti-angiogenic, and is it perhaps the first member of a new type of regulatory growth factor family?

Transgenic mice bearing a gain-of-function *fps/fes* oncogene have such an excess of vasculature that they can readily be recognized at birth by their bright red appearance (Greer *et al.*, 1990). What role does the normal transmembrane receptor counterpart of this oncogene play in regulating angiogenesis? Transgenic mice expressing the v-*jun* oncogene show a marked inability to heal wounds and a characteristic response to regulatory cytokines (Schuh *et al.*, 1990; Vanhamme *et al.*, 1993). Is the inhibition of wound healing in part due to an altered response to angiogenic factors?

Much speculation has centered on oncogenes and tumor suppressor genes (Brem and Klagsbrun, 1993; Bouck, 1993) and their role in angiogenesis. The question arises, however, will the information gained from oncogenes and suppressor genes permit us to identify the normal processes by which angiogenesis is regulated?

Perhaps the most compelling information on angiogenesis and anti-angiogenesis will come from knowledge gained from studies of the early mammalian embryo. As the embryo develops, angiogenesis is turned on and off accurately and with an exquisiste sense of timing. In turn, blood vessels from in the placenta, radiate from the extraembryonic membranes to surround the embryo, and arise in each organ bed as each organ rudiment grows and differentiates. At precisely the right time, angiogenesis is halted, the vascular system is complete, endothelial cell proliferation is reduced from the most rapid to the slowest of all cell types. And yet, even when turned off, the endothelium is ready to respond once again, transiently stimulated by cyclic signals in the uterus or the hair follicle, only to turn off again when the signals are changed. Perhaps it is through understanding the normal processes underlying the development of the vascular system that we will be able to make the greatest progress towards successful control of the angiogenic response.

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